INVESTIGATIONS INTO THE MEASUREMENT OF NITROGEN FIXATION IN FRESHWATER LAKE SEDIMENTS.

Rosemary Sylvester-Bradley

Ph D Thesis
University of Edinburgh
March 1976

I declare that this thesis has been composed by myself, and that the results described are my own, except for the mass spectrometric analyses in Appendix II, and some of the acetylene reduction assays in Experiment 1, Chapter 4.

Mud! Mud! Glorious mud!
Nothing quite like it for cooling the blood!
So follow me follow,
Down to the hollow,
And there let us wallow
In glorious mud!

From "The Hippopotamus"
(Flanders & Swann, 1952).

ACKNOWLEDGMENTS

I am grateful for the help of the following:

Dr. A.J. Holding, my supervisor,

The Institute of Terrestrial Ecology, and their staff at 12 Hope Terrace, Edinburgh, and Sir David Montgomery, owner of Loch Leven, who made facilities and equipment at Loch Leven available to me,

The Freshwater Biological Association, Windermere, and their staff, in particular Dr. Vera Collins and Mr. Graham Hall, who made possible the smooth progress of the experiments I carried out in their laboratory,

Miss Liz Field, Miss Libby Bailey, Drs. Alex and Claire Macleod and Miss Marilyn Elliott, who lent me their cars for the purpose of sampling trips,

Dr. Keith Jones and Mr. David Bangs who made a gas chromatograph at Lancaster University available to me,

Dr. Tony Haystead of the Hill Farming Research Organization, Bush, who advised me on the ^{15}N-enrichment experiment, and carried out the mass spectrometric analyses,

Dr. Barry Jupp of St. Andrews University who advised me on the Potamogeton experiments, and provided me with some of the material,

Dr. Johanna Dobereiner for her collaboration in Experiment 1, Chapter 4, and her infectious enthusiasm,

The Natural Environment Research Council for financial support,

all my friends and family who enabled me to remain reasonably sane, particularly my father Prof. Peter C. Sylvester-Bradley who read and criticized the manuscript, and Miss Liz Field, whose help in preparing the drawings was invaluable,

with many of whom I had useful and stimulating discussions, and who often went to considerable inconvenience to help me.

ABSTRACT

The acetylene reduction assay was tested, and found not to measure nitrogen fixation in lake sediments quantitatively for three reasons:

1) Ethylene absorption by the sediment was inhibited by acetylene. It was therefore not possible to measure and subtract non-nitrogenase-mediated ethylene production from the results.

2) Ethylene absorption by sediment was found to be inhibited under similar conditions as ethylene uptake by a culture of Methylomonas albus (BG8). Nitrogen fixing methane oxidizers which do not reduce acetylene may therefore be active in the sediment.

3) The rate of ethylene production in acetylene reduction assays of lake sediment increased at up to at least 1.0 atm C_2H_2 in the gas phase, and was higher if the samples were incubated under shaking than under static conditions. The rate of ethylene production by unshaken sediment was inhibited by N_2. No ^{15}N-enrichment of unshaken sediment from $^{15}N_2$ was detected. This may have been due to the slow diffusion of gases into the sediment.

Rates of ethylene production in acetylene reduction assays of a range of freshly sampled sediments from Loch Leven (Kinross), and Esthwaite Water and Grasmere (English Lake District) were measured and compared. Loch Leven sediment produced more ethylene if it was black than if it was brown, and under anaerobic than aerobic conditions of incubation, and at higher temperatures. Numbers of endospore-forming acetylene-reducing rods counted by an anaerobic most probable number technique were higher in black than brown sediment, and in non-pasteurized than pasteurized sediment. However, other acetylene-reducing bacteria were also present. The highest rates of ethylene production from freshly sampled sediments were observed with Esthwaite and Grasmere sediments two weeks after the overturn, which may have been due to aerobic organisms.

Roots of Potamogeton filiformis and P. pectinatus from Loch Leven reduced acetylene at much higher rates than the leaves or root-free sediment. Cultures of microaerophilic acetylene-reducing spirilla which grow in nitrogen-free media were isolated from P. filiformis roots which reduced most acetylene at 0.02 atm O_2 (90 nmoles C_2H_2 /g dry roots/h).

CONTENTS

CONTENTS continued

CHAPTER 1. INTRODUCTION: PERSPECTIVES, IMPORTANCE AND METHODS OF STUDYING NITROGEN FIXATION IN FRESHWATER LAKES, WITH PARTICULAR REFERENCE TO THE BACTERIA IN THE SEDIMENTS.

All lakes are temporary phenomena. In geological terms, a lake has an origin, it exists for a greater or lesser length of time, and eventually disappears. The disappearance of a lake is often due to sedimentation, whereby the lake is filled with inorganic and organic sediments and eventually becomes dry land; sedimentation is both a physical and a biological process.

From a biological point of view, lakes can be differentiated according to their trophic status. The trophic status of a lake is considered to be a consequence of the stage it has reached in the process of "eutrophication". Eutrophication is probably best defined as the accumulation of nutrients in lakes. This accumulation process can occur even if the loss and gain of nutrients in the lake by physical processes is evenly balanced. This is because nutrients entering a lake may support the growth of living organisms, and the biological material so produced remains in the lake longer than would the inorganic nutrients themselves. Lakes in early stages of eutrophication are termed "oligotrophic". Later they become "eutrophic". However, the trophic status of a lake does not only result from eutrophication; it is also affected by the type of lake basin and physical conditions to which it is exposed. Thus the individual characteristics of a particular lake must be taken into account when assigning it a trophic status.

The living organisms in any ecosystem can be divided into primary and secondary producers. Primary producers (phototrophic organisms and chemoautotrophic bacteria) can utilize inorganic nutrients. Secondary producers (animals and other heterotrophs) must feed on organic compounds. The quantity of primary production may therefore control that of secondary production, and consequently the supply of inorganic nutrients may control the total biological productivity of an ecosystem. Thus the supply of carbon, nitrogen and phosphorus, which are major constituents of biological tissues, is likely to limit biological productivity. In lakes silicon has also been considered as a possible limiting nutrient because it forms the skeleton of diatoms, and these

organisms are the dominant primary producers in some lakes (Hutchinson, 1957).

Inorganic nutrients are not only supplied to a lake from external sources. They can also be regenerated from organic matter by decomposition and converted from one inorganic form to another by chemical and biological processes within the lake. Carbon and nitrogen can also be exchanged with the atmosphere, for example, by the processes of nitrogen fixation and denitrification. "Nutrient cycles" describe the routes which nutrients take as they are transferred between different parts of the biosphere. The nitrogen cycle in lakes has been discussed by Hutchinson (1957) and Keeney (1973) and involves the interconversion of gaseous nitrogen, organic nitrogen, ammonium nitrogen and nitrate nitrogen. Clearly the rate of nitrogen cycling will be limited if one of these chemical forms is unavailable.

The importance of biological nitrogen fixation in lakes. The supply of nitrogen fertilizer to any agricultural crop is often the limiting factor to its productivity. Biological nitrogen fixation is a potential solution to the world problem of obtaining sufficient nitrogen fertilizer for agriculture. There is essentially unlimited nitrogen in the atmosphere, and biological nitrogen fixation can be driven by the chemical energy produced by photosynthesis from sunlight, which is also unlimited, as far as man is concerned. Chemical processes which carry out the same reaction must be supplied with energy from other limited sources and are therefore less economical.

Man's ability to control primary productivity by agriculture does not only depend on obtaining sufficient nutrients. It is also important that the production is of the desired type. The nitrogen cycle is thus of qualitative as well as quantitative importance because different organisms assimilate different chemical compounds of nitrogen preferentially. In natural lakes also certain types of organism are of more use to man than others. For example, the fish and wildfowl are valuable for recreational purposes, and certain species of fish are more palatable than others. Biological nitrogen fixation could affect the quality of production in freshwater lakes by increasing the growth rates of some plants more than others. Thus in affecting the ecology of primary producers, nitrogen fixation could also affect the fish and

wildfowl populations, even if it were not of overall quantitative significance in the lake.

The effect of nitrogen fixation on lakes may in some cases be actively detrimental. As discussed already, eutrophication can be defined as the accumulation of nutrients in lakes. The activities of man tend to result in the increase of the flow of nutrients into lakes. For example, a certain proportion of artificial fertilizers added to farmland may be leached out of the soil into streams and then freshwater lakes or the sea (e.g. Cooke & Williams, 1970). Similarly, industrial effluents such as blood from slaughter houses, sugar from sugar beet factories, or detergents and domestic sewage contain high levels of nutrients and are sometimes discharged into lakes. These effects may be collectively termed "cultural eutrophication". The more nutrients there are available in a lake, the greater the quantity of primary production is likely to be. Such an increase in primary production increases secondary production, including that of decomposition by bacteria. Since most heterotrophic growth is respiratory, the oxygen in the lake is depleted and the sediment and even the water may become anaerobic. In severe cases of cultural eutrophication only anaerobic bacteria and photosynthetic organisms can grow. Under natural conditions such a severe trophic state is only reached in a lake which has almost disappeared as a result of sedimentation. Thus natural eutrophication is not so undesirable as cultural eutrophication. In that it is a process which adds nitrogen to a system, nitrogen fixation contributes to eutrophication. Thus it can be seen that nitrogen fixation in lakes may have both detrimental and beneficial effects.

Some reported studies on the eutrophication of lakes lead to further conclusions as to whether or not nitrogen fixation in them is important. Biological nitrogen fixation could explain the findings of Vollenweider (1968) that phosphorus is the limiting nutrient in lakes more often than nitrogen. He concluded that to prevent cultural eutrophication it was more important to eliminate phosphorus than nitrogen from streams entering the lake. If nitrogen fixation occurred in a lake it would indeed seem that the elimination of nitrogen from external sources would be an ineffective measure for the prevention of cultural eutrophication.

Mortimer (1938) studied the nitrogen balance of some English Lake District lakes of varying trophic status. He found that the quantities of nitrogen leaving and entering the more oligotrophic lakes were more or less balanced, but that only half the amount of nitrogen which entered eutrophic lakes left in the outflow. The simplest explanation of this observation would be that if occurring at all, the processes of nitrogen fixation and denitrification were evenly balanced in oligotrophic lakes, but that denitrification predominates in eutrophic lakes. If this were the case, denitrification would be an important factor counteracting eutrophication in lakes. Hutchinson (1957 p 846) states that in view of Mortimer's results "in no case is it necessary to postulate very intense (nitrogen) fixation in the lake". However, it is possible that all the nitrogen which disappeared was not lost from the lake but was immobilized in the sediment or the bodies of dead or living organisms, and so was not measured by the methods used. Also, nitrogen compounds in lake water are always being taken up and released in various forms and thus to understand their availability it is necessary not only to know their concentration but also the rate of turnover. Vollenweider emphasized that for this reason the concentration of nutrients in a lake was a less important measure of trophic status than the rate of supply from external sources. Thus it can be seen that the nitrogen balance of a lake is no more than the overall effect of the dynamic processes affecting it. To explain the nitrogen balance it would be necessary to study the rates of the individual processes which convert nitrogen from one form to another, and which transport its various compounds between different parts of the lake.

Factors affecting nitrogen fixation in lakes. Several workers have studied various aspects of nitrogen fixation in lakes and the factors which seem to affect them. Biological nitrogen fixation is mediated by the enzyme nitrogenase which has so far only been found to occur in certain blue-green algae and bacteria. Synthesis of nitrogenase is controlled by feed back inhibition by ammonia in cultures of these organisms (Hardy et al, 1973). Thus it might be

expected that more nitrogen fixation would occur in oligotrophic than in eutrophic lakes. However, in view of evidence to the contrary, Stewart (1969) concludes that in most ecosystems the levels of combined nitrogen are insufficiently high to inhibit nitrogen fixation. Also, because nutrients leaving and entering a lake become bound up in plant and animal tissues, the nutrient concentration in the water may be as low in eutrophic as in oligotrophic lakes. The nitrate concentration in the waters of the eutrophic Loch Leven and Esthwaite Water are below 0.1 ug/ml in the summer (Holden & Caines, 1974; Heron, 1961) which is as low as the average levels in most oligotrophic lakes. On the other hand, the sediments of more eutrophic lakes have higher total nitrogen contents (Mortimer, 1938) but these concentrations are still not apparently high enough to inhibit nitrogen fixation (Keeney, 1973).

Nitrogen fixation is a very energy demanding process and in the same article (1969) Stewart suggests that nitrogen fixation would be most likely to occur in association with photosynthesizing plants which are able to provide sufficient energy in the form of carbohydrates or other respirable substrates. The nitrogen fixed in such associations would also be in a better position to be assimilated by the plants and therefore to increase primary production. However, there is no reason why nitrogen fixation should not occur in other lake habitats rich in energy containing compounds, for example, in the sediments where decomposition of algal cells is taking place. As primary production is greater in eutrophic than oligotrophic lakes, there is likely to be more energy available in them. Thus both heterotrophic and photosynthetic organisms might fix nitrogen at a higher rate in eutrophic than oligotrophic lakes.

This dependence of nitrogen fixation on both energy and nitrogen levels has led to the conclusion that the C:N ratio may control nitrogen fixation. Huser (1965) studied the effect of C:N ratios on nitrogen fixation in soil and found that there was a particular ratio below which nitrogen fixation did not occur (40:1). This is of considerable importance in agricultural systems where the soil can be ammended with straw or other similar material to increase the C:N ratio. However, in a natural system such as lake sediment, changes in levels of available carbon and nitrogen are not so easy to control. In these systems it is

necessary to know the natural C:N ratio, the measurement of which presents practical problems: measurements of total carbon and nitrogen levels do not show whether or not they would be available to nitrogen fixers. For this reason the results of experiments in which glucose and ammonium were added to lake sediments (Appendix I) were inconclusive. In fact it is easier to measure rates of nitrogen fixation than carbon and nitrogen availability: the fact that nitrogen fixation occurred in a particular sediment would indicate that the carbon in it was available and that the nitrogen levels were not inhibitory to nitrogenase synthesis. Similarly, measurements of rates of nitrogen fixation might be expected to be related to measurements of rates of decomposition in lake sediments. Thus, although it may be possible to determine the effect of the C:N ratio on nitrogen fixation in lake sediments, this would be difficult to investigate and in any case not so relevant as in agricultural systems.

Methods for studying nitrogen fixation in lakes. There are various techniques available for the study of the ecology of nitrogen fixing organisms. Nitrogen fixing bacteria can be counted by growing them in nitrogen free media. However, Hill & Postgate, (1969) have warned of the danger of assuming that all bacteria which grow in nitrogen free media are nitrogen fixers, because of the ability of some non-nitrogen-fixing bacteria to grow on traces of ammonium and other nitrogen compounds. The most probable number technique suggested by Campbell & Evans (1969) avoids this problem because the organisms are only counted if they reduce acetylene, a reaction specific to nitrogen fixers (e.g. Hardy et al, 1973). It seems that any nitrogen fixer for which a selective enrichment medium can be found could be counted by this method. However, all the usual errors involved in counting bacteria by indirect enrichment culture methods apply: nitrogen fixing bacteria cannot be identified by direct microscopic examination but only by their characteristics when growing in pure culture, and any counting method which relies on growth in artificial media only counts organisms which will grow in that medium. These organisms are not necessarily the ones which are active in the natural habitat and thus the count may give a false impression. Total counts of bacteria in soil made by direct microscopic examination may be several orders of magnitude higher than total counts made by culture methods (e.g. Hayes & Anthony, 1959). The results of counts of any

bacteria must therefore be interpreted with caution.

There are also methods available for the measurement of the rate of nitrogen fixation. Those most commonly used are to measure ^{15}N-enrichment from $^{15}N_2$, and the acetylene reduction assay. The $^{15}N_2$ method is more direct, but also more expensive and laborious than the acetylene reduction method. The best approach is therefore probably a judicious combination of the two.

A further useful approach to the study of nitrogen cycling in lakes is to add ^{15}N-labelled nitrogen compounds to sediment systems and study their interconversion (e.g. Keeney et al, 1971; Chen et al, 1972). This makes it possible to put studies of the individual processes of the nitrogen cycle into perspective.

There are various problems involved in the use of these methods for the study of the ecology of nitrogen fixing bacteria. As mentioned already, certain species of blue-green algae and bacteria can fix nitrogen. Blooms of nitrogen fixing blue-green algae often occur in eutrophic lakes in the summer, and it is well established that they can contribute significant amounts of nitrogen to the nitrogen cycle (e.g. Dugdale & Dugdale, 1962; Goering & Neess, 1964). However, it is not always obvious which organisms in a sample are responsible for the nitrogen fixing activity in it. Algae can be colonized by nitrogen fixing bacteria (e.g. Head & Carpenter, 1975), whose activity could be falsely attributed to the algae if they were not detected. Similarly, nitrogen fixing activity which occurs in the dark cannot always be assumed to be due to bacteria, because blue-green algae have been shown to be able to fix nitrogen heterotrophically (Fay, 1965; Watanabe & Yamamoto, 1967; Cox & Fay, 1969). On the other hand, the nitrogen fixing organisms shown to be present in a sample are not necessarily fixing nitrogen; they could be in a metabolically inactive state or growing on other nitrogen compounds. Obviously it is necessary both to measure the rate of nitrogen fixation and to determine the numbers of different nitrogen fixers present in a sample. If the conditions under which nitrogen is fixed are the same as those required by the nitrogen fixer found to be most abundant in the sample, it can be inferred that this organism is responsible. If the changes in numbers of a certain nitrogen

fixing organism in a habitat are found to correlate with changes in nitrogen fixing activity this is also a good indication that that organism is responsible. Even this approach is not perfect because nitrogen fixation may not always be growth dependent (Dobereiner, in press).

Further problems are encountered because of the great physiological diversity of nitrogen fixers. For example, the nitrogen fixing bacteria include the photosynthetic bacteria, the microaerophilic spirilla and methylobacteria, the facultatively anaerobic Klebsiella, which until recently (Hill, 1975) were thought only to fix nitrogen under anaerobic conditions, and obligately anaerobic Clostridia and Desulfovibrio species. (For a full list of the known types see Postgate, 1971a). There may also be other organisms with a yet undiscovered ability to fix nitrogen. Thus there is a potentially unlimited range of habitats in which nitrogen fixation might occur, and it would be necessary to know all the conditions which may favour nitrogen fixation in a given ecosystem before generalizations as to its significance could be made. In addition to their relationship to carbon and nitrogen levels mentioned already all known nitrogen fixers do have the characteristic of oxygen sensitivity in common. Even strict aerobes such as Azotobacter grow better under low oxygen tensions when fixing nitrogen (Dalton & Postgate, 1968). This is presumed to be due to the extreme oxygen sensitivity of the enzyme nitrogenase, which is irreversibly damaged if exposed to air during purification (Hardy et al, 1973). However, since in most habitats there are microsites containing low oxygen concentrations this probably does not restrict the distribution of bacterial nitrogen fixation very extensively. Many nitrogen fixing bacteria are adapted to overcome their oxygen sensitivity. For example Azotobacter produces slime, and high respiratory activity enables it to fix nitrogen under aerobic conditions, albeit less efficiently (Postgate, 1971b). Heterocysts in blue-green algae are thought to have a similar protective role (Fay et al, 1968; Stewart et al, 1969).

If it is intended to express the rate of nitrogen fixation in quantitative terms, the conditions under which the measurement applies must be specified very precisely, as is the case with all measurements of enzyme activity; such a measurement without conditions is of limited

value. As it is difficult to specify all the conditions in lake sediments which might be affecting nitrogen fixation, measurements of rates of nitrogen fixation are difficult to interpret in quantitative terms even in laboratory systems. Nevertheless non-quantitative results do have some importance.

Most of the work on nitrogen fixation in lake sediments reported in the literature seems to have been aimed either at determining the most abundant nitrogen fixers or at measuring the amount of nitrogen being fixed in order that a "nitrogen budget" for the lake can be constructed. In the experiments described in this thesis it was attempted to investigate both qualitative and quantitative aspects of nitrogen fixation in lake sediments, and to interpret the results in the light of the discussion in this chapter. In Chapter 2 it is shown that the acetylene reduction assay is of limited use for the measurement of nitrogen fixation in lake sediments. Further experiments were conducted in order to investigate the diversity of habitats within lake sediments in which nitrogen fixation occurs (Chapters 3 and 4). It was found that there was in fact considerable diversity among sediment samples, and more study is needed into different sediment habitats, and into the organsims responsible for nitrogen fixation in them. It might then be possible to generalize as to the quantity of nitrogen fixed in lake sediments: it is essential to study the quality of nitrogen fixation in lake sediments as well as to measure it if the significance of the measurements are to be understood.

CHAPTER 2. CRITICISM OF THE ACETYLENE REDUCTION ASSAY FOR THE MEASUREMENT OF NITROGEN FIXATION IN FRESH WATER LAKE SEDIMENTS.

2-1 Introduction

Dilworth (1966) and Schollhorn & Burris (1966) independently discovered that, among other triple bonded carbon and nitrogen compounds, the nitrogen fixing enzyme nitrogenase will also reduce acetylene. Stewart et al (1967) and Hardy et al (1968) introduced acetylene reduction and measurement of the product ethylene by gas chromatography, as a routine method for the measurement of nitrogen fixation. Since then it has been used extensively on cell free, single cell, agricultural and natural ecosystems. Hardy et al (1973) have written a comprehensive review of the known uses and limitations of the technique. They feel that if used with proper caution ("in all cases specific assay systems should be calibrated with a nitrogen based method" p. 52) the acetylene reduction assay can give a quantitative measure of nitrogen fixation. However, Bergersen (1970) warned that this might not be so in ecosystems where several different nitrogen fixing systems co-exist . Another method of showing whether rates of acetylene reduction are a quantitative measure of nitrogen fixation is to test whether they possess the characteristics of an enzyme catalysed reaction. In the experiments described in this chapter, certain of these characteristics were tested in a lake sediment system, in order to define the limits of measurement of the technique. Some of the requirements which must be fulfilled if the acetylene reduction assay is to be used as a quantitative measure of nitrogen fixation in lake sediments are listed below:

1) The rate of acetylene reduction should be constant and measured prior to any change which might occur with time.
2) Any change in ethylene concentration not due to nitrogenase must be measured and taken into account.
3) Acetylene reduction should be universal to all systems containing active nitrogenase.
4) The level at which acetylene concentration ceases to limit the rate of acetylene reduction must be known.

5) Acetylene should be a preferred substrate to nitrogen, or if it is not, all nitrogen gas should be eliminated from the system.

The form of the acetylene reduction assay tested was based on the system recommended by Hardy et al (1968), i.e. a sealed container in which the sample is placed under a specified gas phase to which the acetylene is added. The ethylene concentration in the gas phase is measured at time intervals, and calculated to give nanomoles ethylene produced per unit sample per hour. As the aim of the investigation is to test whether the rate of ethylene production can be assumed to represent the rate of acetylene reduction, the results are termed "rates of ethylene production", rather than "rates of acetylene reduction". All the incubations were carried out in the dark.

2-2 Investigations into the variation of rate of ethylene production with time.

Introduction. When determining the rate of an enzyme catalysed reaction, it is essential to measure the initial rate, because side effects may cause a change of rate with time. For example, Hardy et al (1968) found that with soybean nodules the rate of acetylene reduction was only constant for 1-2 hours, after which it decreased. On the other hand Rinaudo et al (1971) found that the rate in plant free soils increased with time, and concluded that derepression of nitrogenase synthesis by the product ammonia was occurring.

If the substrate is supplied in sufficient concentration to saturate the enzyme, and no inhibitors are present, the initial rate will be constant for a given enzyme concentration under specific conditions. The initial rate of the reaction is therefore a measure of enzyme activity.

If, however, the substrate is not supplied in sufficient concentration to saturate the enzyme, this will limit the rate of the reaction. It is possible that in some habitats the availability of gaseous nitrogen limits the rate of nitrogen fixation. If this is the case, the acetylene concentration used in acetylene reduction assays must saturate the same proportion of enzyme sites as would be saturated by gaseous nitrogen in the natural habitat. If this is not done, a

PLATE 1

Jenkin's corer and freshly sampled core at Loch Leven North Deep. Black sediment is overlain by brown sediment in the core.

false estimate of the rate of nitrogen fixation will be obtained. It is usually assumed that the availability of gaseous nitrogen does not limit nitrogen fixation, and acetylene is supplied at 10 or 20% v/v in the gas phase. This is considerably more than the concentration required to saturate the nitrogenase in many of the systems which are reported in the literature (see section 2-5).

In the experiments described in this section it was attempted to show whether or not lake sediment reduced acetylene, and if so whether the rate of ethylene production was constant. The fulfillment of this criterion alone is not sufficient to show that the acetylene reduction assay is a quantitative measure of nitrogen fixation, because the rate might be limited by some other constant factor. However, it is essential that the rate is linear if it is to be measured.

Materials and Methods. The sediment was collected in an Ekman Grab (described by Edmondson & Winberg, 1971) from Loch Leven North Deep, mixed, and stored at 4°C, or in a Jenkin's Corer (described by Mortimer, 1971) and used less than five hours after sampling. The Jenkin's Corer collects a core of sediment 7cm in diameter and up to 25cm deep, together with a sample of lake water from immediately above the sediment (see Plate 1). The Ekman Grab collects about 3 litres of mud from approximately 0-15cm depth in the sediment. Samples collected in the Jenkin's Corer are less disturbed than those in the Ekman Grab. Sub-cores of the sediment samples were taken using 5ml plastic disposable syringes of which the narrowing end of the barrel had been sawn off. Three 2ml samples from each sub-core were then extruded onto 3cm square pieces of heavy duty plastic sheeting. The sediment was rolled up in the plastic and thus transferred into narrow necked 17ml glass bottles without touching the glass which it would otherwise stick to. Inside the bottles the plastic unrolls and leaves the sediment exposed to the atmosphere. Pierced metal caps with rubber seals were screwed down tightly onto the bottles, and the bottles were flushed for 10 minutes with oxygen free nitrogen (OfN_2) through a manifold fitted with syringe needles. Each bottle was vented through a second needle. When exposure of the sediment to oxygen was undesirable, the transfer to the bottles was carried out in a gas tight glove bag filled with OfN_2; the sawn-off

plastic syringes can be stoppered with rubber bungs to exclude air, although they cannot be stored for long because air can permeate the plastic. The bottles were then flushed with acetylene for three further minutes, sealed with liquid rubber sealer, (e.g. aquarium sealer available from F.W. Woolworths & Co. Ltd.) and incubated at the specified termperature. Bottles without acetylene were also included so that any ethylene produced independently of nitrogenase would be detected. In the experiments shown in Figures 2-1 and 2-2, different bottles were used for each individual gas sample. Owing to the slow rate of ethylene production it was not possible to detect changes in ethylene concentrate in less than four hours. Therefore the long incubation time (24 hours) was unavoidable.

The gas phase was analysed as follows: the Pye 104 gas chromatograph (flame ionization detector; N_2 carrier gas flow rate 34ml/min; hydrogen 15lb/in^2; air 20lb/in; oven temperature 50°C) was calibrated for ethylene on the appropriate attenuation settings (x50 or x100) by means of replicated dilutions of pure ethylene in sealed flasks of known volume. Provided that the gas chromatograph was zeroed as described in the instruction manual before each use, the relationship of ethylene injected to peak height was linear over a wide range of attenuations. The amount of ethylene contaminant in the acetylene was calculated and subtracted where necessary.

Results and Discussion. The results of preliminary experiments (not shown) were very erratic. It was deduced that leakage of oxygen into the bottles or oxidation of the samples during transfer inhibited acetylene reduction and that the removal of more than one sample from a bottle caused errors due to depletion of the gas phase. Also, if rubber seals from metal caps which had previously been exposed to high levels of ethylene were used, they released ethylene during the incubation, as described by Kavanagh & Postgate (1970). If particular care was taken to avoid oxidation of the sediment, and new rubber seals were used, however, reliable results such as those described below were obtained.

Some examples of the many time curves which were constructed are shown in Figures 2-1 and 2-2, together with an example of the calibration

FIG 2-1

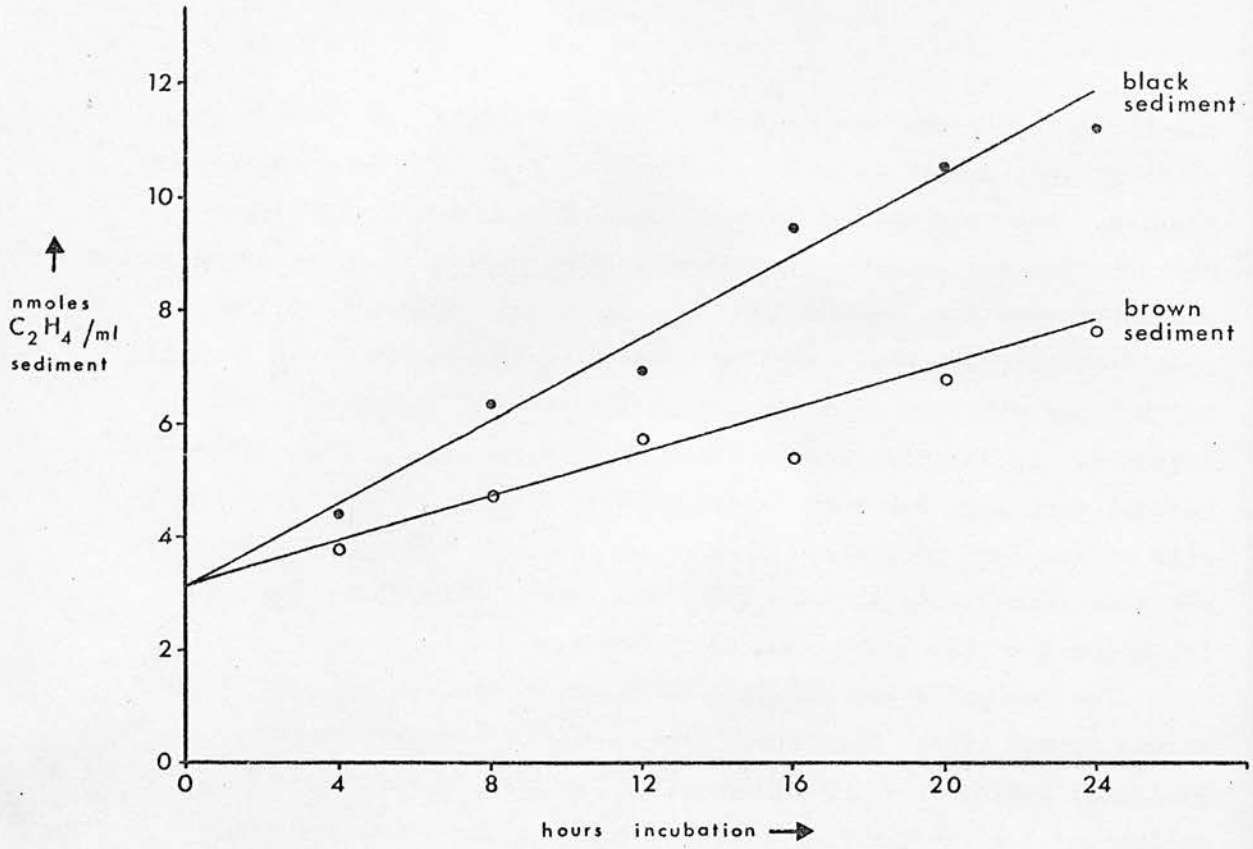
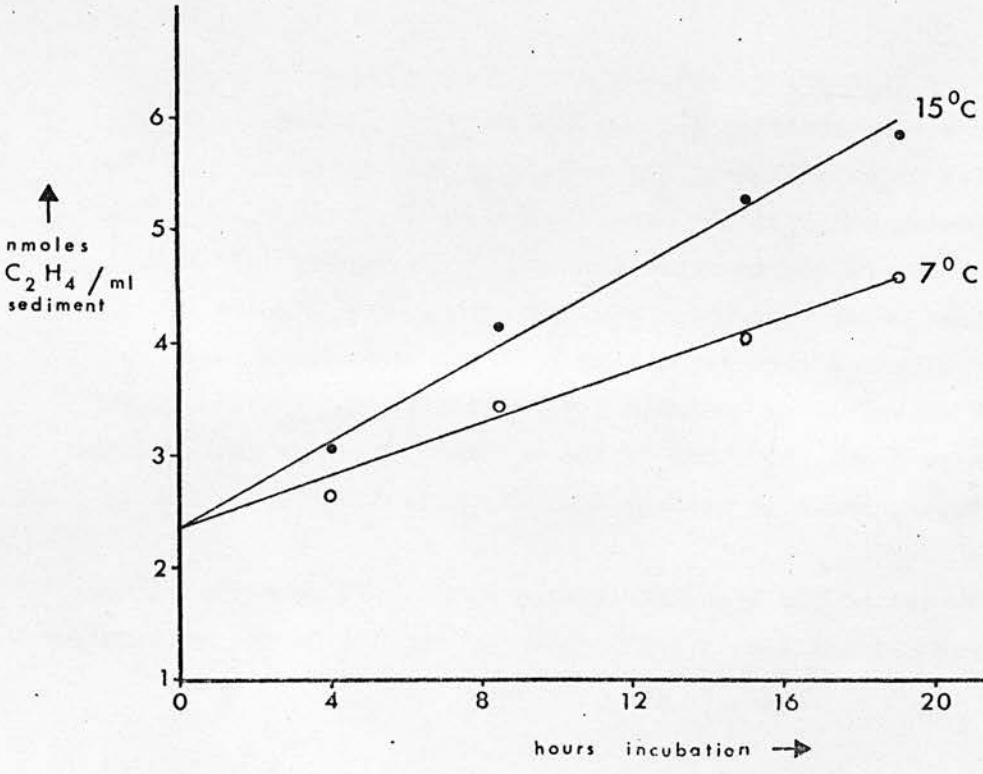

nmoles
C_2H_4 /ml
sediment
0
2
4
6
8
10
12
0
4
8
12
16
20
24
hours incubation
black
sediment
brown
sediment

FIG 2-2


nmoles
C_2H_4 /ml
sediment
1
2
3
4
5
6
0
4
8
12
16
20
hours incubation
15°C
7°C

Example of calibration of gas chromatograph. F values used in Chapter 2 were calculated from means of 4 such calibrations.

Dilutions of ethylene were made by injecting pure ethylene into a sealed flask (Flask 1), and then injecting a gas sample from Flask 1 into a second similar flask (Flask 2). A 0.5 ml sample from Flask 2 was injected into the gas chromatograph and the ethylene peak height measured. This procedure was repeated four times, keeping the original ethylene in the flasks. The ethylene thus accumulated in the flasks.

Calibration:

$V_1 \cdot V_2$	total in Flask 2	GCU	GCU (difference)	y	y (average)	V_2	F
0.96	0.96	1000	1000	0.960			
0.96	1.92	2050	1050	0.914	0.900	311	1.97×10^{-4}
1.28	3.20	3500	1450	0.883			
1.60	4.80	5400	1900	0.842			

where v_1 = volume injected into Flask 1
v_2 = volume injected into Flask 2
GCU = gas chromatograph units (ethylene peak height)
$y = v_1 \cdot v_2 / 1000$ GCU
V_2 = volume of Flask 2
F = nmoles ethylene/GCU

$$F = \frac{y}{V_2} \times \frac{20.8}{V_1}$$ (where V_1 = volume of Flask 1)

FIGURE2-1

Ethylene production by black or brown sediment from Loch Leven North Deep incubated under static anaerobic conditions with 1.0 atm C_2H_2 at 15°C (nanomoles ethylene/ml). Points are means of three replicates. Average range = 16.4% (range of individual values calculated as percentage of means, and averaged).

FIGURE 2-2

Ethylene production by black sediment from Loch Leven North Deep incubated under static anaerobic conditions at 7°C or 15°C with 1.0 atm C_2H_2. Points are means of three replicates. Average range = 15.5% (calculated as above).

of the gas chromatograph. The black and brown sediment in Figure 2-1 was from the same North Deep (Loch Leven) Jenkin's core, in which brown sediment occurred in a 10cm layer on top of black sediment. It can be seen that acetylene reduction by the sediment did occur, that the rate of ethylene production was linear, and was affected by the sample or temperature used. Thus, from these preliminary results there is no reason to suppose that acetylene reduction is not a quantitative measure of nitrogen fixation in lake sediments. Some further tests were nonetheless carried out.

2-3 Investigations into the ability of sediment to produce and remove ethylene independently of nitrogenase.

Introduction. Some fungi, bacteria and plants can produce ethylene in the absence of acetylene (e.g. Bird & Lynch, 1974; Abeles, 1973; Primrose, 1975; Primrose & Dilworth, 1975). Methane oxidizers can oxidize ethylene (de Bont & Mulder, 1974; Whittenbury et al, 1975), and ethylene absorption occurs in some soils (Abeles et al, 1971; Yoshida & Ancajas, 1971) and lake water (Flett et al, 1975). de Bont (1975) has isolated an organism (possibly a Mycobacterium sp) which appears to be able to grow with ethylene as an energy source. It is necessary to test whether any of these reactions occur in lake sediments, and if so, what effect they may have on the acetylene reduction assay.

Ethylene absorption was investigated in some Scottish soils and in Loch Leven North Deep sediment. Some of this work has been described previously (Sylvester-Bradley et al, 1974 see enclosed reprint). In these experiments, although ethylene absorption was prevented by autoclaving, no difference between the effects of aerobic and anaerobic treatments on ethylene absorption was observed. This was surprising because if ethylene absorption was biologically mediated it would be affected by these conditions. It seemed possible that the oxygen in the aerobic treatment did not properly pervade the sediment. Therefore the experiment has been repeated using more thoroughly aerated sediment, and is reported in this section.

FIG 2-3

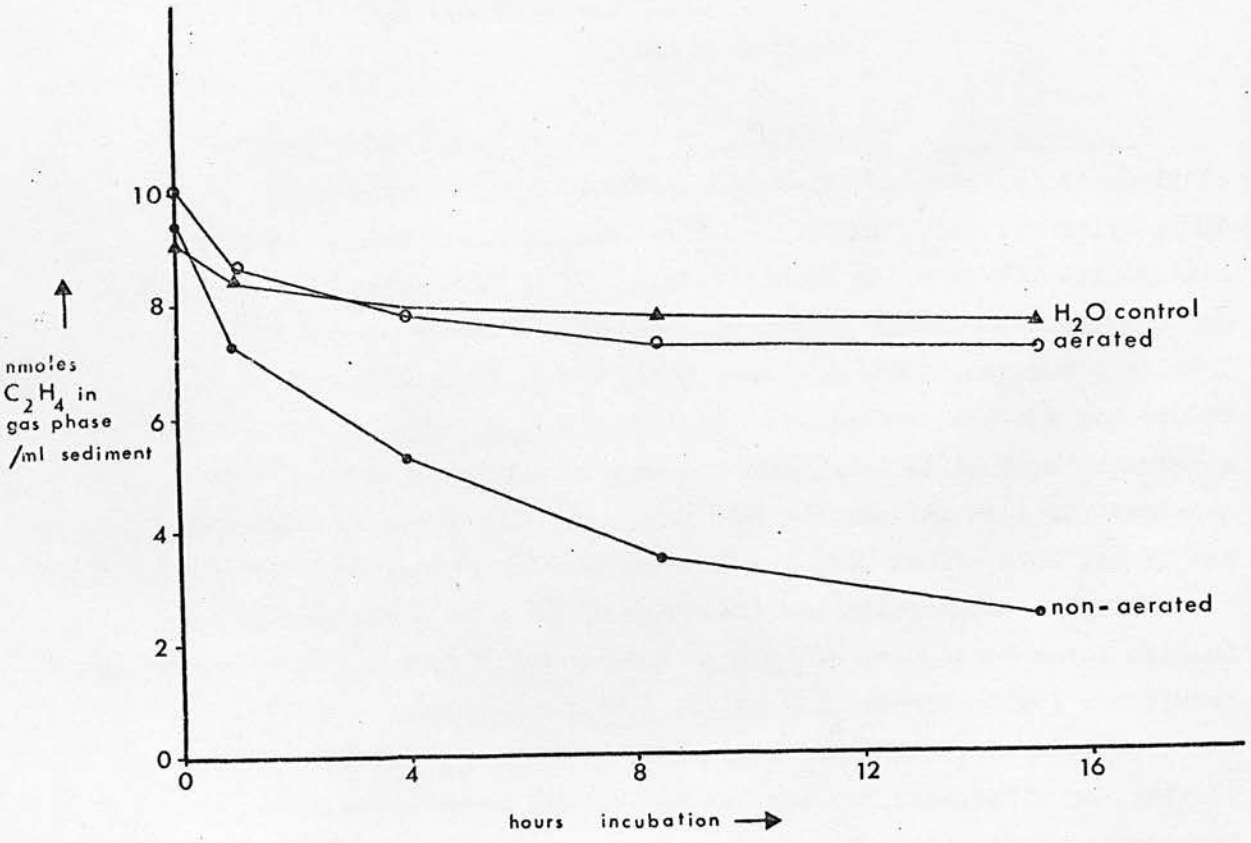

nmoles
C_2H_4 in
gas phase
/ml sediment
10
8
6
4
2
0
0
4
8
12
16
hours incubation
H_2O control
aerated
non-aerated

Figure 2-3

Ethylene absorption by aerated and non-aerated black sediment from Esthwaite Water, and water control, incubated anaerobically at 15°C. Points are means of three replicates.

Materials and Methods. One of two cores which had been collected from Esthwaite Water (English Lake District) during the summer stratification period was aerated at 10°C for a week by bubbling air through the overlying water in the Jenkin's core tube. The other core was sealed and incubated without aeration, also at 10°C. The surface layer of the aerated core, which had been black, became brown. The sediment in the non-aerated core remained black throughout. Three 2ml samples of surface mud were taken from each core with a wide mouthed pipette and put into narrow necked 17ml bottles. A set of bottles containing 2ml water was included as a control. The bottles were sealed and flushed with OfN_2 as described in section 2-2. 0.5ml of an approximately 10^{-3} dilution of ethylene in OfN_2 was then added to each bottle. They were incubated at 15°C. Ethylene peak height was measured on the gas chromatograph as described in section 2-2, at the time intervals shown in Figure 2-3. Great care was taken not to contaminate any of the syringes, manifold bottles etc. with acetylene, as even small traces of acetylene inhibit ethylene absorption (Sylvester-Bradley et al, 1974).

Results and Discussion. It can be seen (Figure 2-3) that aeration of the sediment inhibited ethylene absorption. Moreover, experience showed that the sediment lost its ability to remove ethylene on prolonged storage. These observations, together with the fact that autoclaving prevents ethylene removal (Sylvester-Bradley et al, 1974) are evidence that ethylene removal by lake sediment is biologically mediated.

Flett et al (1975) observed ethylene absorption by lake water, but did not note that it was inhibited by acetylene. Although, as was previously concluded (Sylvester Bradley et al, 1974), acetylene prevents the direct interference of ethylene absorption with the acetylene reduction assay, ethylene absorption may prevent the detection of non-nitrogenase-mediated ethylene production. Therefore ethylene absorption is more likely to cause an overestimation than an underestimation of acetylene reduction rates.

2-4 Universality of acetylene reduction to all systems containing active nitrogenase.

Introduction. Until recently it was thought that acetylene

reduction was "uniquely universal" to all systems containing active nitrogenase (Hardy et al, 1973). No system not containing nitrogenase has yet been shown to reduce acetylene to ethylene. However, de Bont & Mulder (1974) and Whittenbury et al (1975) have found that acetylene reduction is not universal to all systems containing active nitrogenase: methane oxidizers able to fix nitrogen are unable to reduce acetylene when growing on methane. Methane oxidizers can occur in large numbers in lake sediments (up to 10^8/ml sediment water slurry; Whittenbury et al, 1975). It is therefore important to determine whether nitrogen fixing methane oxidizers are active in sediments in which it is intended to measure rates of nitrogen fixation by the acetylene reduction assay.

Methane oxidizers can decompose ethylene, and are microaerophilic when fixing nitrogen. Whittenbury et al (1975) suggest that methane oxidation by methane oxidizers is inhibited by acetylene, whereas methanol oxidation is not. The energy required for nitrogenase activity is therefore unavailable to methane oxidizers growing on methane if acetylene is present. If this were the case, nitrogen fixation by methane oxidizers growing on methane could be demonstrated by $^{15}N_2$ uptake but not by acetylene reduction, whereas they would reduce acetylene and fix $^{15}N_2$ when growing on methanol. This effect was observed by de Bont & Mulder (1974), although they proposed a different explanation. The ethylene removal by lake sediment described already (section 2-3) was inhibited by acetylene and under aerobic conditions. It therefore seems that it might be due to methane oxidizers. Some experiments were carried out to compare ethylene uptake and its inhibition by a pure culture of Methylomonas albus (BG8) and by lake sediment. Similarities between the two would be at least partial evidence that methane oxidizers were present and active in the sediment. An alternative method would then be needed to measure their nitrogen fixing activity.

Materials and Methods. Washed suspensions of BG8 grown on NMS medium (nitrate mineral salts medium as described by Whittenbury et al, 1970) diluted in phosphate buffer (pH 6.8: 1.0g Na_2HPO_4 and 0.8g KH_2PO_4 per litre) to an optical density of approximately 130 were kindly provided by Mrs Eleanor Hogg. 0.1ml of the cell suspension was added to 4.9ml of the same buffer or 4.7ml of the same buffer plus 0.2ml 10^{-3} Molar

FIG 2-4

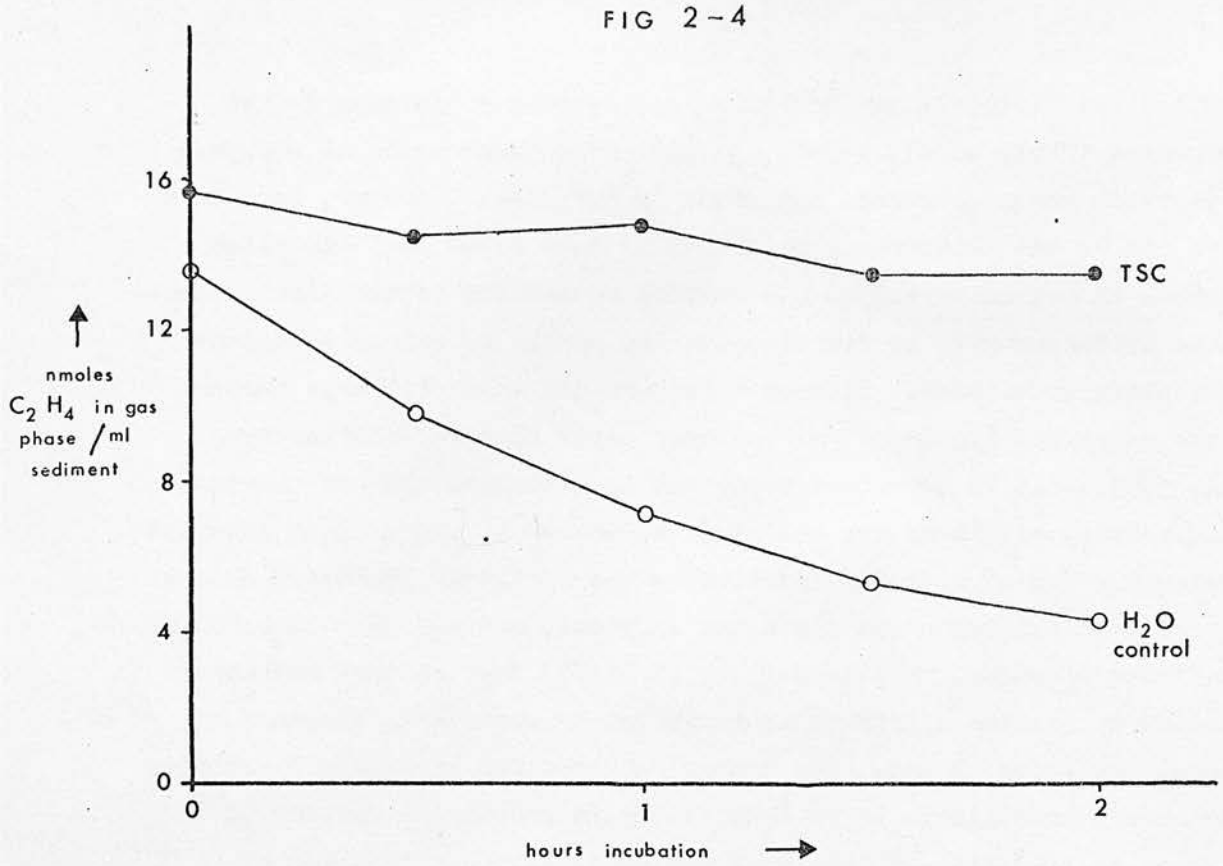

16
12
8
4
0
nmoles C_2H_4 in gas phase / ml sediment
TSC
H_2O control
0
1
2
hours incubation

FIG 2-5

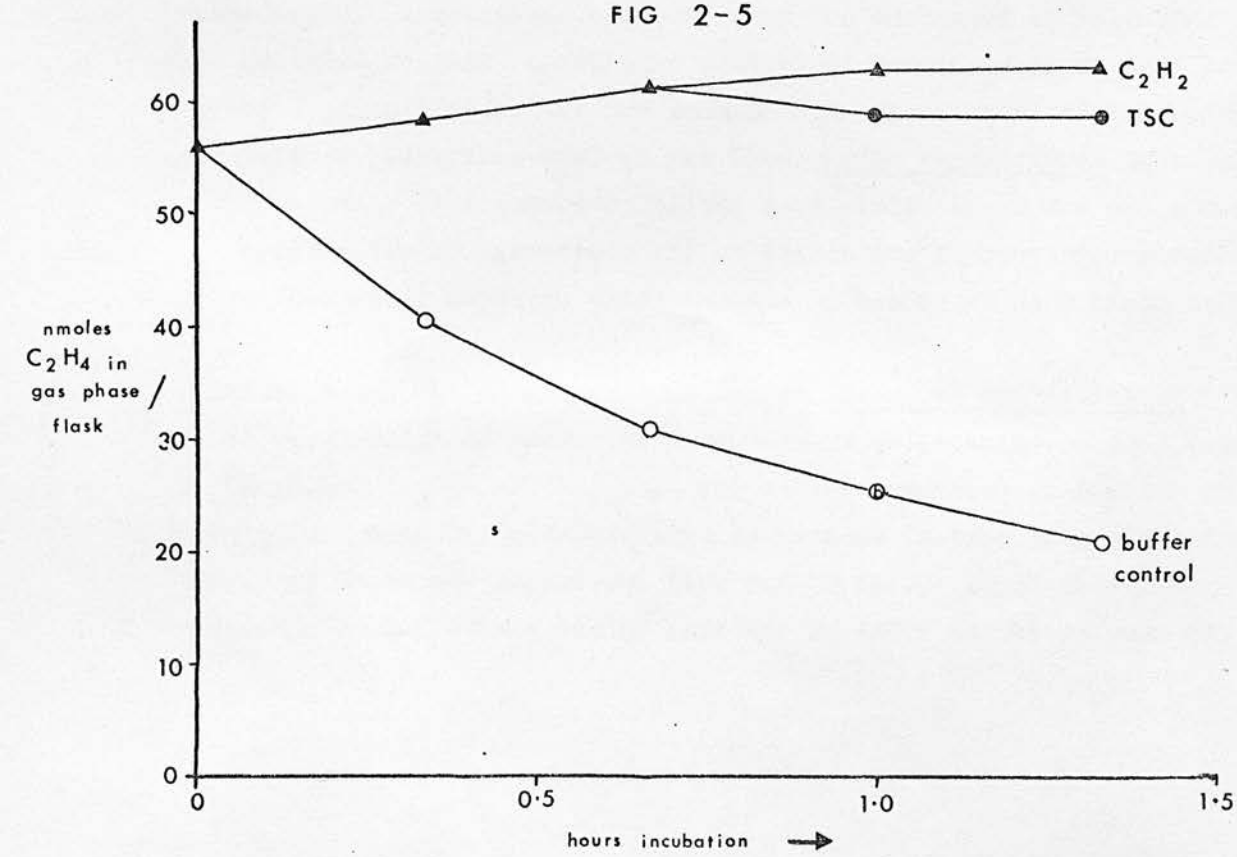

60
50
40
30
20
10
0
nmoles C_2H_4 in gas phase / flask
C_2H_2
TSC
buffer control
0
0·5
1·0
1·5
hours incubation

Figure 2-4

Ethylene absorption by black Loch Leven North Deep sediment with added water (control) or aqueous solution of thiosemicarbazide (TSC) to give a concentration of 10^{-3}M. Incubated anaerobically at 37°C without shaking. Points are means of three replicates.

Figure 2-5

Ethylene uptake by washed cell suspensions of Methylomonas albus (BG8) diluted in phosphate buffer (pH 6.8) with 4 x 10^{-5} M thiosemicarbazide (TSC), without TSC but with 1.6 x 10^{-4} atm C_2H_2 in the gas phase, or without either TSC or C_2H_2 (control). Incubated with 0.04 atm O_2 at 30°C on a wrist action shaker.

thiosemicarbazide (TSC) in 25ml round bottomed flasks. (Ethylene and methane oxidation by methane oxidizers is inhibited by various compounds and is discussed by Hubley et al (1975) and Hubley (1975). It seems that thiosemicarbazide may act by chelating copper ions which may be a component of the methane oxygenase system). The flasks were sealed with new Suba-seals (size 37 red rubber with sleeve obtainable from William Freeman & Sons Limited, Suba-seal Works, Barnsley, Yorkshire), flushed out with OfN_2 through the manifold as described in section 2-2, and 4% v/v O_2 was added. 0.5ml 10^{-3} ethylene in OfN_2 was added to all the flasks and 0.5ml 10^{-3} acetylene in OfN_2 to one treatment. The flasks were incubated on a wrist action shaker at $30^{o}C$.

Sediment samples were collected from Loch Leven North Deep in an Ekman Grab, transferred into bottles and flushed with OfN_2 as above. Either TSC to give a concentration of 10^{-3} Molar, or the same volume of distilled water were added by syringe, followed by 0.5ml 10^{-3} ethylene in OfN_2. The bottles were incubated statically at $37^{o}C$.

All treatments were carried out in triplicate.

Results and Discussion. It can be seen in Figures 2-4 and 2-5 that TSC inhibited ethylene disappearance both in BG8 and in lake sediment. Even the low concentration of acetylene used inhibited ethylene removal by BG8, and since the acetylene peak height did not change, this cannot have been due to acetylene reduction. However, although this shows that there is a similar inhibition pattern in BG8 and lake sediment, in further experiments BG8 oxidized more ethylene under aerobic than semi-aerobic (4% O_2) conditions, whereas ethylene removal by lake sediment is inhibited by aeration (section 2-3). This discrepancy could be due to the fact that methane oxidizers are only microaerophilic when fixing nitrogen. All my attempts to grow BG8 in semi-solid nitrogen free media have failed, and it seems that this organism may not be a nitrogen fixer. A further experiment to show whether ethylene oxidation is microaerophilic in an N_2 grown methane oxidizer is needed here. Thus it is still possible that non-nitrogen-fixing organisms such as those isolated by de Bont (1975) might be responsible for some or even all of the ethylene removal by lake sediments. Further experiments could be designed to show whether the ability of non-nitrogen-fixing ethylene

oxidizers to decompose ethylene is affected by acetylene, and to determine the numbers of these organisms and methane oxidizers in the sediment. In any case, it seems that it would be dangerous to assume that the acetylene reduction assay measures the total nitrogen fixing activity of lake sediments; such assays are likely to under-estimate nitrogen fixation in sediments where methane oxidizers are active. It is possible however, that they could be used to distinguish between the activity of nitrogen fixing methane oxidizers and that of other nitrogen fixers in the sediment.

2-5 Attempt to determine the level at which acetylene concentration ceases to limit the rate of acetylene reduction in lake sediments.

Introduction. The initial velocity of an enzyme catalysed reaction increases with substrate concentration up to a point at which the substrate saturates all the active enzyme sites. The rate of the reaction then becomes constant for a given enzyme concentration and set of conditions. In order to obtain a correct measurement of nitrogenase activity it is essential that the enzyme is saturated with acetylene to the same extent as with nitrogen in the natural habitat (see section 2-2). In the experiments described here, an attempt is made to determine the concentration of acetylene necessary to saturate the nitrogenase in lake sediments. This is usually expressed as the Michaelis constant (Km) which describes the affinity of the enzyme for its substrate. (Km = substrate concentration at which the initial velocity of the reaction is half the maximum velocity, at specified enzyme concentration and conditions). The apparent Km for acetylene reduction by a synthetic soil system ammended with 2% glucose was 6×10^{-3} atm C_2H_2 (Brouzes & Knowles, 1973), and most other systems except the rice rhizophere gave a similar value (Hardy et al, 1973). However, Spiff & Odu (1973) found that acetylene reduction by Beijerinckia indica in shaken liquid cultures increased up to 0.74 atm C_2H_2. If an inhibitor of the reaction is present, it may alter the response of the velocity of the reaction to substrate concentration in one of several ways depending on its mode of inhibition. When determining the Km of a purified enzyme it is relatively easy to ensure that no

inhibitors are present. In lake sediment it is not so easy, and the possible effect of inhibitors must be taken into account when interpreting the results.

Materials and Methods. Sediment was collected from Loch Leven North Deep in an Ekman Grab, stored, sampled and transferred into 17ml bottles as described in section 2-2. Either helium or OfN_2 was used as the flushing gas. Where the experiments included shaking treatment, 1ml of distilled water saturated with the appropriate flushing gas was added to all the bottles. In Figure 2-6, various acetylene concentrations were established in the gas phase. To obtain approximately 60% acetylene the bottles were flushed with pure acetylene and then 10ml of the gas phase was removed and replaced by 6ml of He or OfN_2. 40% acetylene was obtained by the opposite procedure. The lower acetylene concentrations were obtained by adding pure acetylene by syringe to the anaerobic bottles. In Figure 2-7, the differing initial amounts of ethylene were obtained by adding a range of ethylene dilutions to bottles containing the same initial amounts of acetylene. The bottles were shaken on a wrist action shaker where specified. Incubation was at 30°C.

Both acetylene and ethylene peak heights were measured on the gas chromatograph. It is these measured levels which are discussed and plotted on the graphs, not the volumes added to the bottles. Ethylene peak height showed a linear response to the amount injected and was calibrated as described in section 2-2. However, acetylene was measured on the 50×10^4 attenuation, which is at the least sensitive extreme of the attenuation range. On this attenuation peak height did not respond linearly when increasing amounts of acetylene were injected. Calibration curves for acetylene peak height against acetylene injected, and acetylene injected against ethylene contaminant in the acetylene were therefore constructed for each experiment. The ethylene contaminant for each acetylene level was thus determined and subtracted from the total measured ethylene.

Results and Discussion. The effect of increasing acetylene concentration in the gas phase under shaking and static conditions with oxygen free nitrogen as the flushing gas after twelve hours incubation

FIG 2-6

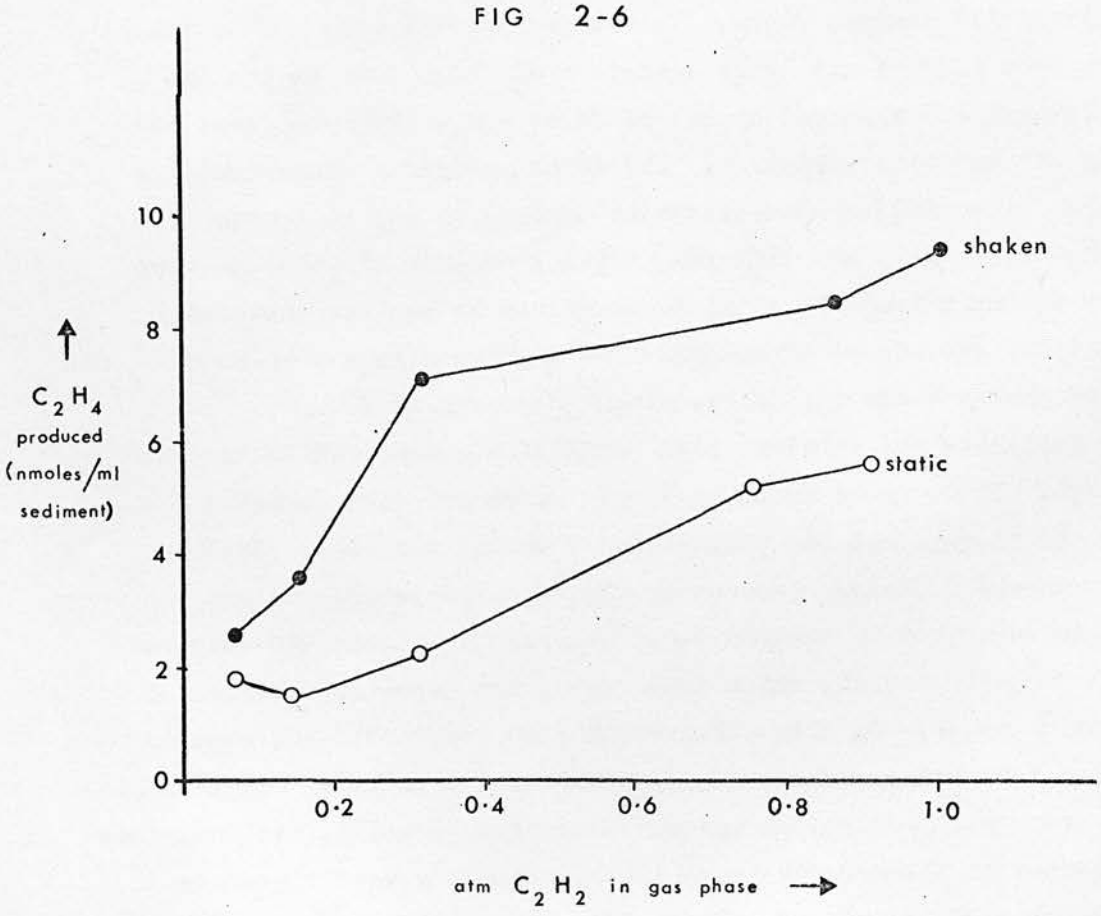

10
8
6
4
2
0
shaken
static
0·2
0·4
0·6
0·8
1·0
C2H4 produced (nmoles/ml sediment)
atm C2H2 in gas phase

Figure 2-6

Ethylene produced by black diluted Loch Leven North Deep sediment during 12 hours anaerobic incubation at 30°C with OfN_2, under static or shaken conditions, with a range of acetylene concentrations in the gas phase. Points are means of three replicates. Average range = 35% (range of individual values calculated as percentage of means, and averaged).

TABLE 2-1

Ethylene produced in acetylene reduction assays of black sediment from Loch Leven North Deep. Conditions as specified.

2-1a - 0.4 atm C_2H_2; 5.5h static incubation at 30°C

Flushing gas / nmoles C_2H_4 produced/ ml sediment	Of N_2	He
individual values	0.83 0.93 0.96 1.05 0.67	0.90 1.09 1.28 1.09 0.96
average	0.88	1.06

2-1b - 0.4 atm C_2H_2; 5.5h shaking incubation at 30°C

Flushing gas / nmoles C_2H_4 produced/ ml sediment	Of N_2	He
individual values	1.53 1.85 1.41 1.25 1.28	1.15 1.47 1.21
average	1.46	1.28

2-1c - 16 h shaking incubation at 30°C; He flushing gas

atm C_2H_2: / nmoles C_2H_4 produced/ ml sediment	0.2	1.0
individual values	2.14 5.05 4.73 3.77	10.54 11.82 11.18 13.10
average	4.17	11.66

is shown in Figure 2-6. Similar results were obtained from several such experiments analysed after different incubation times. It can be seen that the results are not as would be expected if the nitrogenase in the sediment was demonstrating Michaelis kinetics. Ethylene production is only half as high from static as from shaken sediment, and does not reach a plateau even at 100% acetylene. These results could be due to the rates of acetylene reduction being limited by the rate of gas diffusion across the gas sediment interface: it would be expected that higher acetylene concentrations and the shaking of the samples would increase the rate of gas diffusion. However, there are other possible explanations.

For example, in the above experiment (Figure 2-6) nitrogen was used as the flushing gas. In cell free preparations of nitrogenase, nitrogen gas does not affect the rate of acetylene reduction because acetylene is a preferred substrate to nitrogen except at very low concentrations of acetylene (Schollhorn & Burris, 1967). This is a desirable characteristic as it means that nitrogen gas does not need to be eliminated from the system when measuring rates of acetylene reduction. On the other hand, in some systems (e.g. soybean nodules: Hardy et al, 1968) even 20% acetylene does not inhibit nitrogen fixation. Consequently it is necessary to flush all nitrogen out of these systems with an inert gas (e.g. helium or argon); it is possible that the results in Figure 2-6 are at least partially due to the use of nitrogen as the flushing gas.

The results of further experiments using helium as the flushing gas are shown in Table 2-1. In Table 2-1a more ethylene does seem to have been produced with helium as the flushing gas than with nitrogen. However, when the bottles were shaken (Table 2-1b) there was no apparent difference between the two treatments. Thus it would seem that nitrogen gas does inhibit acetylene reduction by lake sediments, but that flushing out the bottles with helium removes the nitrogen gas dissolved in the sediment when the bottles are shaken. It is also implied that the diffusion of nitrogen gas across the sediment gas interface is very slow unless the sediment is shaken.

In Table 2-1c the results of an experiment designed to show whether Michaelis kinetics are demonstrated by sediment shaken with helium can be seen. However, even under these conditions more than twice the amount of ethylene was produced with 100% acetylene than with 20% acetylene. If

FIG 2-7

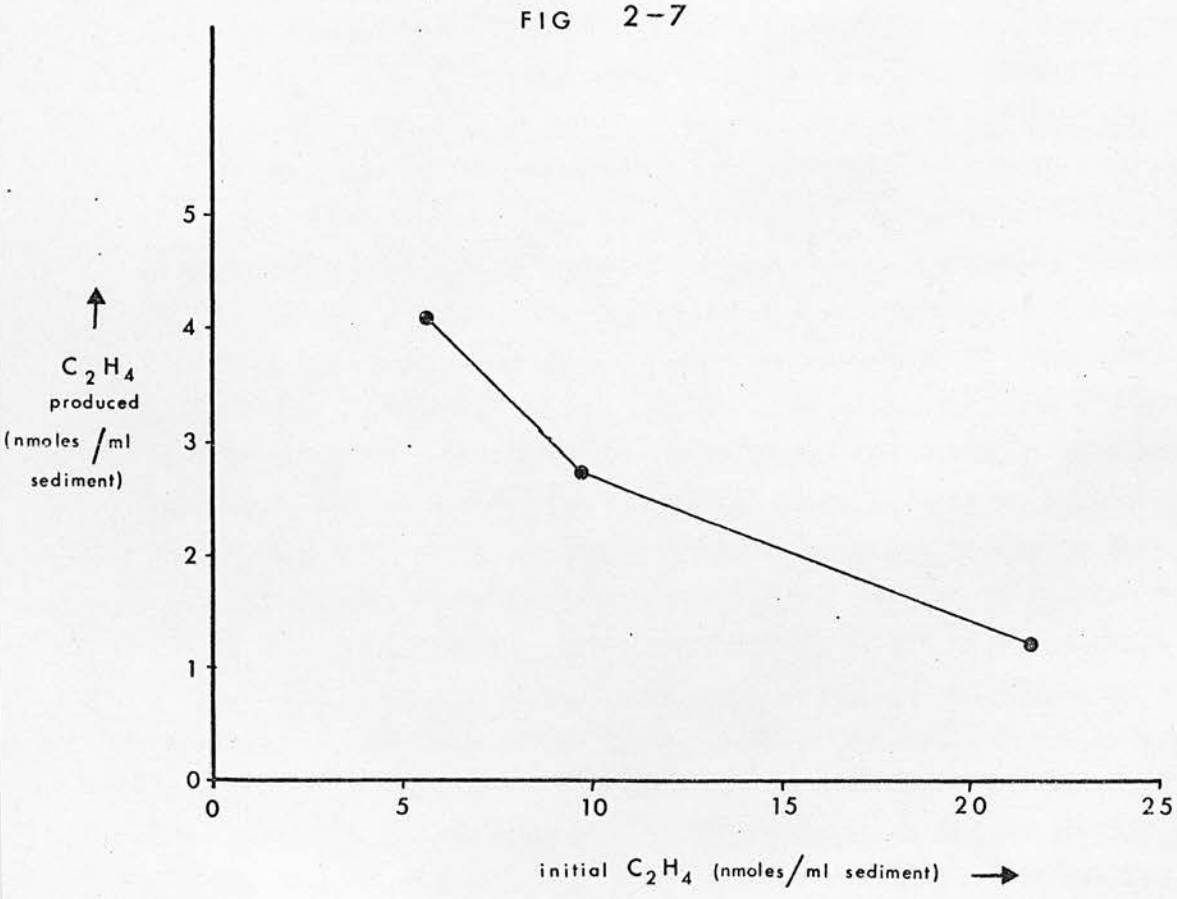

C_2H_4 produced (nmoles/ml sediment)
0
1
2
3
4
5
0
5
10
15
20
25
initial C_2H_4 (nmoles/ml sediment)

Figure 2-7

Ethylene produced by black Loch Leven North Deep sediment during 8 hour anaerobic static incubation at 15°C, with 0.4 atm C_2H_2 and a range of initial C_2H_4 concentrations in the gas phase. Points are means of three replicates.

this is plotted on a graph, the lowest possible acetylene concentration at which the maximum rate of ethylene production occurs is 0.4 atmospheres. The "Km" would therefore be at least 0.25 atmospheres. It seems unlikely that this is the case, and it can only be concluded that nitrogen gas is not the only factor which prevents the demonstration of Michaelis kinetics in Figure 2-6.

Apart from the acetylene concentration, one other such factor could be the increasing level of ethylene contaminant in the acetylene rather than the acetylene itself. However, in an experiment carried out to test this by adding different amounts of ethylene to bottles containing the same amount of acetylene, it was found that ethylene production was inversely related to initial ethylene concentration (Figure 2-7). Thus in Figure 2-6 the concentration of the acetylene in the gas phase must itself be affecting the rate of ethylene production, most probably by its concentration dependent diffusion into the sediment. This conclusion is supported by the results of an attempt to measure ^{15}N-enrichment of unshaken sediment from $^{15}N_2$ (method described in Appendix II), which detected no enrichment after three days even though the same sediment produced 0.37 nmoles ethylene/ml sediment /h in acetylene reduction assays, and the sensitivity of the method was to .001 atom % excess ^{15}N above a natural ^{15}N level of .336%. As nitrogen gas is sixty times less soluble in water than acetylene, it would be expected to diffuse even more slowly into the sediment.

It seems necessary to explain the results in view of the observation that the rate of ethylene production is linear, at least when incubated with 100% acetylene statically (section 2-2). If the explanation is that the rate of diffusion of acetylene into the sediment limits the rate of acetylene reduction, this rate of diffusion would depend on the concentration of acetylene in the gas phase, which is constant, and therefore it would be linear. However, the results in Figure 2-7 show that higher initial levels of ethylene in the gas phase inhibit ethylene production, so the rate of ethylene production would be expected to slow down as it proceeds. Therefore, if such feedback inhibition does occur, it would be necessary to postulate yet a third mechanism which causes an increase of rate with time to account for the linearity of ethylene production. For example, some workers report the existence of a lag before the onset of ethylene production

(e.g. Dobereiner et al, 1973). In the absence of a definite explanation it does seem most likely that the rate of diffusion of acetylene into lake sediment limits the rate of acetylene reduction causing considerable underestimation of nitrogenase activity, particularly if the samples are incubated statically. Waterlogged soil was found by Rice & Paul (1971) to need much higher than usual $^{15}N_2$ application levels to saturate its nitrogenase. This supports the hypothesis that there is some factor peculiar to lake sediment and waterlogged soil, such as their high viscosity, which prevents the rapid diffusion of gases through them or between them and the gas phase.

Flett et al (in press) found that the ethylene produced in acetylene reduction assays on lake water did not reach equilibrium with the gas phase unless the bottles were shaken vigourously at the end of an experiment. The ethylene present in both gas and liquid phases was then calculated according to Henry's partition law. The authors do not mention that they had any difficulty in saturating the nitrogenase with acetylene. This may be because acetylene diffuses more rapidly into water than sediment. However, it does seem that the rate of gas diffusion may be a more widespread problem in acetylene reduction assays than was previously appreciated.

It is clear that for quantitative acetylene reduction assays on lake sediments a system must be devised whereby it can be ensured that gas diffusion rates are not limiting. Even when the sediment was diluted with water and shaken on a wrist action shaker as described in this section, the extent to which it mixed with the gas phase was minimal. Macgregor et al (1973) used glass beads to improve gas diffusion in their samples. In my experience, the sediment is too viscous for sufficiently thin distribution over the surface of the beads. It is possible that if the sediment was diluted sufficiently for it to become completely non-viscous this problem would be solved. This would be the most important experiment to do next as it would also test the hypothesis that it is gas diffusion that is limiting the rate of acetylene reduction. However, it must be borne in mind that in thus destroying the structure of the sediment its effect on the nitrogen fixing organisms it contains may be changed considerably.

It seems from the results in Table 2-1 that nitrogen gas inhibits acetylene reduction in lake sediments. Thus in these experiments under-

estimation of the rate of nitrogen fixation would occur where any nitrogen gas was present in the bottles. It would also occur if denitrification was taking place; denitrified nitrogen would interfere with acetylene reduction assays in the same way as nitrogen in the gas phase. Indeed, Chen et al (1972) found that $^{15}NO_3$ added to sealed sediment systems was converted to $^{15}NH_4$, which could be due to the $^{15}NO_3$ being denitrified to $^{15}N_2$ and subsequently fixed by nitrogen fixers. Terry & Nelson (1975) also concluded that denitrification could occur in lake sediments. The problem of inhibition of acetylene reduction by nitrogen gas in lake sediments might be overcome if the sediment was saturated with acetylene. However, if denitrification does occur in lake sediments, it would be necessary to take it into account when measuring rates of nitrogen fixation by the method using ^{15}N-enrichment from $^{15}N_2$, because denitrified nitrogen would not initially be labelled, but would nonetheless be fixed.

Conclusions. Attempts to determine the Michaelis constant for acetylene reduction by lake sediment revealed that it was unrealistically high. In the absence of any other obvious reason for this it seems that it is due to the diffusion of acetylene into the sediment being so slow that it limits the rate of acetylene reduction. It is suggested that it might be possible to overcome this problem by diluting the sediment until it is completely non-viscous and shaking it to ensure that the partition of gases between the gas and liquid phases is at equilibrium. The total ethylene could then be calculated by Henry's law as suggested by Flett et al (in press).

2-6 Summary and Conclusions

Rates of ethylene production from lake sediment incubated with acetylene remained constant for at least 24 hours (section 2-2), and ethylene absorption by lake sediment is inhibited by acetylene (section 2-3). However, it is not possible to measure non-nitrogenase-mediated ethylene production (section 2-3), and nitrogen fixing but non-acetylene-reducing methane oxidizers may be present in the sediment (section 2-4). Also, gas diffusion rates limit the rate of acetylene reduction (although this problem might be overcome), and under these conditions denitrification in

the sediment would inhibit acetylene reduction. It would probably also inhibit ^{15}N-enrichment from $^{15}N_2$ (section 2-5).

In view of these conclusions it seems that it would be almost impossible to ensure that the acetylene reduction assay is a quantitative measure of nitrogen fixation in lake sediments, or even to define the limits of measurement by the technique. The observations of Brouzes & Knowles (1971) and Ormeland & Taylor (1975) that acetylene inhibits Clostridium pasteurianum and methanobacteria only goes to support this suggestion. It seems that problems might also be encountered using ^{15}N-enrichment from $^{15}N_2$ as a quantitative method, not least because nitrogen gas is sixty times less soluble than acetylene.

This conclusion is not however as serious as it seems. It is a well known problem of microbial ecology that any method of measurement has its drawbacks. The obvious solution is to use more than one method; if several methods agree it is likely that they are measuring a natural event and not an experimental artifact. Furthermore, quantitative measurement is not the only aim of scientific investigations; it is also important to obtain a qualitative evaluation of the system. As was shown in Chapter 1, qualitative aspects of nitrogen fixation in lake sediments would be worth investigating. The acetylene reduction assay was used for this purpose in the experiments described in Chapters 3 and 4.

CHAPTER 3. SOME INVESTIGATIONS INTO THE QUALITATIVE ASPECTS AND DISTRIBUTION OF NITROGEN FIXATION IN FRESHWATER LAKE SEDIMENTS.

3-1 Introduction

Several attempts have been made to assess the quantitative significance of nitrogen fixation by means of the acetylene reduction assay (described by Hardy et al, 1968 & 1973) in sediments of both freshwater (Howard et al, 1970; Keirn & Brezonik, 1971; Macgregor & Keeney, 1973; Macgregor et al, 1973) and estuarine (Brooks et al, 1971; Marsho et al, 1975; Whitney et al, 1975) ecosystems. In these publications the results are calculated to give a more or less tentative estimate of the amount of nitrogen fixed in the sediment per year, and the figures thus obtained are related to any other data concerning nitrogen transport and transformations in the particular lake or estuary. Until these data on sediments were published it was thought likely that blue-green algae in lake water fixed more nitrogen than bacteria either in the sediment or the water, and several authors have reported investigations into the size of the blue-green algal contribution (e.g. Dugdale & Dugdale, 1962; Horne & Fogg, 1970; Rusness & Burris, 1970; Horne & Viner, 1971; Stewart et al, 1971). There is still some disagreement in the literature as to whether or not the quantity of nitrogen fixation in sediments contributes significantly to the nitrogen cycle in lakes. For example, ~~Brouzes~~ Knowles (in press) suggests that it may well do so, whereas Marsho et al (1975) conclude that it does not. This may be partly because there are differences between the particular ecosystems under investigation. However, in view of the demonstration that the acetylene reduction assay does not measure the total quantity of nitrogen fixed in Loch Leven sediment (Chapter 2), it would seem that in any case further work is needed before a definite conclusion can be drawn. Further studies would also be needed into the availability of molecular nitrogen in the sediment which might limit the rate of nitrogen fixation, and also into the fate of nitrogen once it is fixed. For example, it is not known whether it is mineralized and transferred to primary producers in lake water, or whether it is grazed in the organic form by the zoobenthos. On the other hand the fixed nitrogen might accumulate in the sediment or be denitrified and evolved as gaseous nitrogen to the atmosphere, and thus have no effect

on production in the lake.

Various items of information are available as to the qualitative nature of sediment nitrogen fixation. For example, Collins (1960) isolated nitrogen fixing bacteria from the sediment surface of some Lake District lakes, Gambarian (1958) isolated Azotobacter spp. and Clostridium pasteurianum from the sediments of Lake Sevan, Werner et al (1974) isolated Klebsiella pneumoniae and Enterobacter aerogenes from marine sediments and Whittenbury et al (1975) isolated nitrogen fixing methane oxidizers from lake sediments. Werner et al (1974) and Brooks et al (1971) concluded that anaerobic bacteria are responsible for sediment nitrogen fixation. Macgregor & Keeney (1973) found that the rates of acetylene reduction were higher in the sediment of hard-water than softwater lakes and Marsho et al (1975) discovered seasonal variation in acetylene reduction rates, a peak occurring in the autumn. Howard et al (1970) concluded that temperature was not a significant factor affecting sediment nitrogen fixation. Whitney et al (1975) found that nitrogen fixation expressed per m^2 sediment decreased with depth in the sediment.

The experiments described in this chapter were designed to derive some further qualitative information about sediment nitrogen fixation, in particular the species of organisms responsible and the types of sediment in which they are most active, as such qualitative information is essential for subsequently making quantitative measurements. Nitrogen fixation in the rhizosphere is described elsewhere (Chapter 4), and this chapter is therefore only concerned with root free sediment. The acetylene reduction assay was used because even though it does not measure the total quantity of nitrogen fixed, it is likely that variations in the rates of ethylene production are a consequence of variations in nitrogenase activity. It is also a cheap and simple method so that a wide range of samples can be tested. However, it is necessary to impose some restrictions on the interpretation of the results. For example, it is sometimes assumed in published papers that nitrogen fixation in lake sediments is due to bacteria. However, it is possible that a matt of blue-green algae could develop on the surface of the sediment of lakes where the light penetration is adequate. If the incubations are carried out in the dark and the sediment samples are from below the surface it seems unlikely that phototrophic organisms would be active.

However, blue-green algae cannot be entirely eliminated from consideration because they can fix nitrogen when growing heterotrophically (Fay, 1965; Watanabe & Yamamoto, 1967; Cox & Fay, 1969). The interpretation of the results is further restricted by the fact that the samples were only taken from a surface layer of sediment about 25 cm deep from three eutrophic lakes from a temperate climate. Also, the assays probably exclude the activity of any nitrogen fixing methane oxidizers (section 2-4), are affected by the slow diffusion of gases through the sediment and across the gas-sediment interface, and are inhibited by gaseous nitrogen (section 2-5). Ethylene production in the absence of acetylene cannot be determined because the sediment removes ethylene in the absence of acetylene but not in its presence (section 2-3). Thus the term "ethylene production" is a more accurate description of what occurs than "acetylene reduction".

The acetylene reduction assay was applied to a range of freshly sampled sediments with as little variation in technique as possible, although with some differences from the method described in Chapter 2. It is therefore described in some detail in section 3-2. The methodological details, descriptions of samples and environmental conditions pertaining to individual experiments are described in the subsequent sections. Ethylene production rates are linear (section 2-2) and therefore the results are calculated per hour from only one measurement of the ethylene produced per sample. Incubation times were corrected to the nearest quarter hour.

3-2 Standard technique used for acetylene reduction assays.

The samples were collected in an Ekman Grab or Jenkins Corer, which were also discussed in section 2-2. The Jenkins Corer retrieves about 25 cm of mud when sampled through 20 m depth of water, but only about 20 cm through 3 m of water. This is because the corer settles further into sediment under deeper water owing to the greater momentum it has gathered on its journey downwards. The samples were transported back to the laboratory where the water from the corer was siphoned off. Horizontal slices of sediment of the required thickness were made by

the following procedure (except in experiments 7-10 where special equipment was available). A core was clamped to a retort stand fixed to the bench. The metal cover at the base of the core was replaced by a piece of aluminium sheet about 10 cm square which was in turn replaced by a rubber bung which fitted the inside of the core tube exactly. The sediment and the bung were then pushed up to the top of the core using a smaller tube as a piston. Another Jenkins core tube was placed on top of the first one, and the sediment was pushed into it in the required amounts, which were removed by slipping the aluminium sheet between the two core tubes and slicing through the sediment. By this procedure the sediment is necessarily exposed to the atmosphere which may cause it to become oxidized. To prevent this the next steps in the procedure were carried out as quickly as possible. (Although the Jenkins Corer is normally considered to take undisturbed samples of sediment, it was found that during sampling and slicing of the cores a certain amount of mixing between layers did occur: as the sediment is pushed up inside the core tube the more liquid surface layers tend to be dragged down the sides of the deeper sediment).

3 ml samples of sediment were transferred using a 3 ml spoon into pre-weighed, wide necked, one ounce (28 ml) bottles. The bottles were sealed with white rubber Suba-seals (size 47). The rubber seals from inside the metal caps which were supplied with the bottles were removed and a hole was pierced in the centre of each cap. These were screwed down tightly over each Suba-seal, and the gas phase of each bottle was replaced three times by oxygen free nitrogen (OfN_2) in experiments 1-6 and argon in experiments 7-10, after evacuating them to a pressure of less than 3 cm mercury on a manifold fitted with syringe needles and attached to a mercury manometer. This is termed "anaerobic treatment" although some oxygen probably did remain in the bottles. 10 or 20% (v/v) acetylene was added to the bottles by syringe, and the conditions of incubation chosen and applied as described later (sections 3-3 to 3-5). Except where light conditions are specified, incubation was in the dark.

After between 10 and 25 hours incubation the gas phase was analysed on a Pye 104 series 65 gas chromatograph with a flame ionization detector (OfN_2 carrier gas flow rate 30 mls/minute, H_2 15 lbs/in^2, air 20 lbs/in^2, oven temperature 50-100^{o}C (in order to give a retention time of 2 minutes

for ethylene), detector temperature 150°C, using a five foot long column packed with Porapak R). Ethylene peak height was calibrated by injecting a sample of a 10^{-6} dilution of ethylene. Both ethylene and acetylene peak heights were measured so that leakage of gas out of the bottles could be detected (acetylene being used as an internal standard). Results from bottles which had obviously leaked were omitted.

The bottles were re-weighed after the experiments, placed in a drying oven at 100°C for a day, allowed to cool at room temperature and humidity, and weighed again. Wet and dry weights of each sample were calculated (in experiments 1-4 dry weights only were determined).

Nanomoles ethylene produced per gram sediment per hour were calculated as follows:

$$\frac{(GCU \times a \times b \times F) - c}{d \times e}$$

where GCU = gas chromatograph units (i.e. peak height)

F = calibration factor (nmoles ethylene/GCU)

a = gas volume in bottle

b = 2 (i.e. 1/size of gas sample injected)

c = nmoles ethylene contaminant in bottle

d = hours incubation

e = weight of sample (grams).

The calibration factor (F) was calculated as follows:

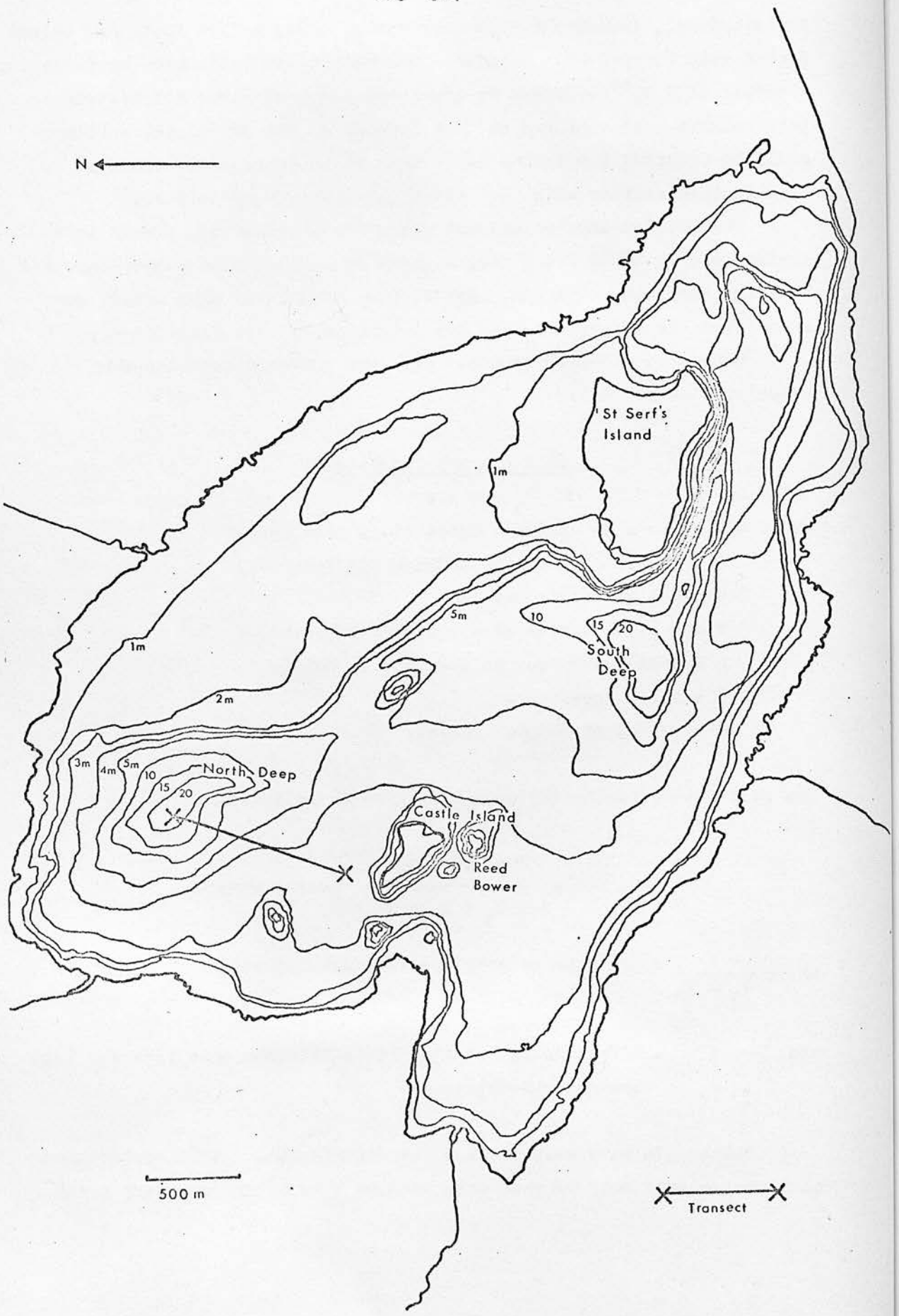
$$1\ GCU = \frac{X \times 0.5 \times 10^9}{Y \times V_1 \times V_2 \times 24{,}000}\ \text{nmoles ethylene,}$$

where $\frac{X}{V_1 \times V_2}$ = dilution of ethylene in flasks of volume V_1 and V_2,

and Y = GCU given by 0.5 ml of this dilution when injected into the gas chromatograph.

Suba-seals were re-used in these experiments. It is recommended that they should only be used once because they adsorb ethylene which

FIG 3-1


N
St Serf's Island
1m
5m
10
15
20
South Deep
1m
2m
3m
4m
5m
10
15
20
North Deep
Castle Island
Reed Bower
500 m
Transect

can then be released during incubations (Kavanagh & Postgate, 1970). However, it was found that the low levels of ethylene adsorbed during these experiments could be removed as follows: the bungs were brought up to pressure (15 lbs/in^2) in an autoclave, and the pressure was then released rapidly. This was repeated three times, and the bungs were then placed in an anaerobic jar and held under vacuum for at least half an hour. Tests showed that this method removed all the ethylene (although not all the acetylene), but care had to be taken that the bungs were not confused with others which had been exposed to higher levels of ethylene. After four or five re-uses the bungs began to leak and were rejected.

3-3 Experiments 1, 2, 3, 4: Black and brown sediment in Loch Leven (Kinross); its distribution and ethylene production rates in acetylene reduction assays.

It was expected that the rate of ethylene production in acetylene reduction assays would be higher in samples containing more organic carbon, both in view of the discussion in Chapter 1, and also the observation that the addition of glucose to sediment stimulated acetylene reduction (Appendix I). A "transect" was therefore marked with buoys across part of Loch Leven (see map Figure 3-1) known to show an increase in organic carbon levels in the sediment (Calvert, 1974). The organic carbon content increases along the transect from 4 to 8% dry weight of sediment with an increase of water depth from 3.5 m to 20 m. Acetylene reduction assays were carried out on freshly collected sediment samples from along the transect and one other site in the loch (South Deep), where the sediment contains over 8% dry weight of carbon.

Methods used, environmental conditions, and appearance of samples.

Experiment 1. On 5th February 1974 (lake temperature 4°C), Ekman grab samples were collected, one at each of four points along the transect line, and numbered 1-4 towards North Deep. The points were identified by taking compass bearings. The sediment in samples 1 and 2 was brown, whereas that in 3 and 4 was blacker. The sediment was transported back to Edinburgh where each sample was exposed to five sets of conditions:

A: light, anaerobic, 23°C;
B: dark, anaerobic, 25°C;
C: dark, 0.05 atm O_2, 25°C;
D: dark, 0.1 atm O_2, 25°C;
E: dark, 0.2 atm O_2, 25°C;

all the bottles being made anaerobic and oxygen subsequently added by syringe. The samples were pre-incubated overnight without acetylene and re-exposed to the various gas mixtures in the morning. Twenty per cent acetylene was then added, and the gas phase was analysed after 24 hours further incubation.

Experiment 2. On 13th March 1974 (lake temperature 5°C) one Jenkins core was collected at each of six 200 m intervals along the 1000 m transect and numbered 1 to 6 towards the deep end (North Deep). There was a layer of brown sediment on the surface of all the cores. This was about 17 cm deep in the cores from under shallow water, and about 12 cm deep in those from under deep water. Beneath the brown sediment was black sediment, which sometimes welled up through channels in the brown sediment to form a layer on the sediment surface (particularly in core 5). The cores were transported back to Edinburgh and each was sliced horizontally to give four 5 cm thick slices. Three approximately 3 ml samples from each slice were transferred into pre-weighed bottles. The bottles were sealed and pre-incubated overnight at 25°C under air. In the morning they were exposed to a fresh atmosphere of 20% O_2, 70% N_2 and 10% acetylene and re-incubated at 25°C. The gas phase was analysed for ethylene after 12-17 hours.

Experiment 3. On 19th June 1974 (lake temperature 15°C) four Jenkins cores were collected from each of the North and South Deeps of Loch Leven (see Figure 3-1). The sediment from the South Deep was more solid and contained more undecomposed organic matter than that from North Deep. The black sediment from each core was separated out into one or two depths, the parts numbered 1 to 7, and four samples of each part were distributed into pre-weighed bottles. Ethylene production per gram dry weight per hour was determined after 15 hours incubation with 10% acetylene in the gas phase at 15°C under anaerobic conditions.

Experiment 4. Several Loch Leven North Deep cores were sampled on 10th July 1974 (lake temperature 15°C). It was found that in addition to a 10 cm layer of black sediment at the bottom of the cores, there was also an approximately 1 cm layer on the top, with 4-5 cm brown sediment inbetween. This did not appear to be due to up-welling of black sediment as was observed in Experiment 2, as there were no channels through the brown sediment. One core was sliced into 13 1.5 cm thick slices and the sediment from each slice was dispensed in triplicate into pre-weighed bottles and incubated with 10% acetylene under anaerobic conditions at 15°C. The gas phase was analysed for ethylene after 25.5 hours.

Results and discussion. The observations concerning the distribution of black and brown sediment in the samples in Experiments 1, 2 and 3 showed that under shallower water there was a layer of brown sediment 15-20 cm deep overlying the black sediment. Under deeper water the layer of brown sediment was much thinner. This suggests that in the region of the North and South Deeps the bulk of the surface 25 cm of sediment was black, whereas in shallower areas of the loch it was mostly brown. The increase in water depth in both North and South Deeps is also accompanied by an increase in sediment organic carbon content (Calvert, 1974). This implies that water depth, blackness and organic carbon content of the sediment may be related is some way. For example, it is possible that in the Deeps, compared with the shallower areas, the sediment is not subjected to so much aeration due to the turbulence of the overlying water. The up-welling of black sediment through the brown, which was particularly marked in Core 5 of Experiment 2, could have been an effect of disturbance by the corer. However, it is also possible that it is a natural phenomenon. For example, the channels through the brown sediment could be created by methane gas bubbling up from the blacker sediment beneath. During the gas chromatographic analysis of ethylene production it was also noted that far more methane was produced from black than brown sediment. In view of the observation by Ormeland & Taylor (1975) that acetylene inhibits methanogenesis, this is likely to have been methane already present in the sediment before the acetylene was added. It does, however, imply that in the loch methanogenesis is greater in black than brown sediment. In Experiment 3 it was also found that

FIG 3-2

(EXPERIMENT 1)

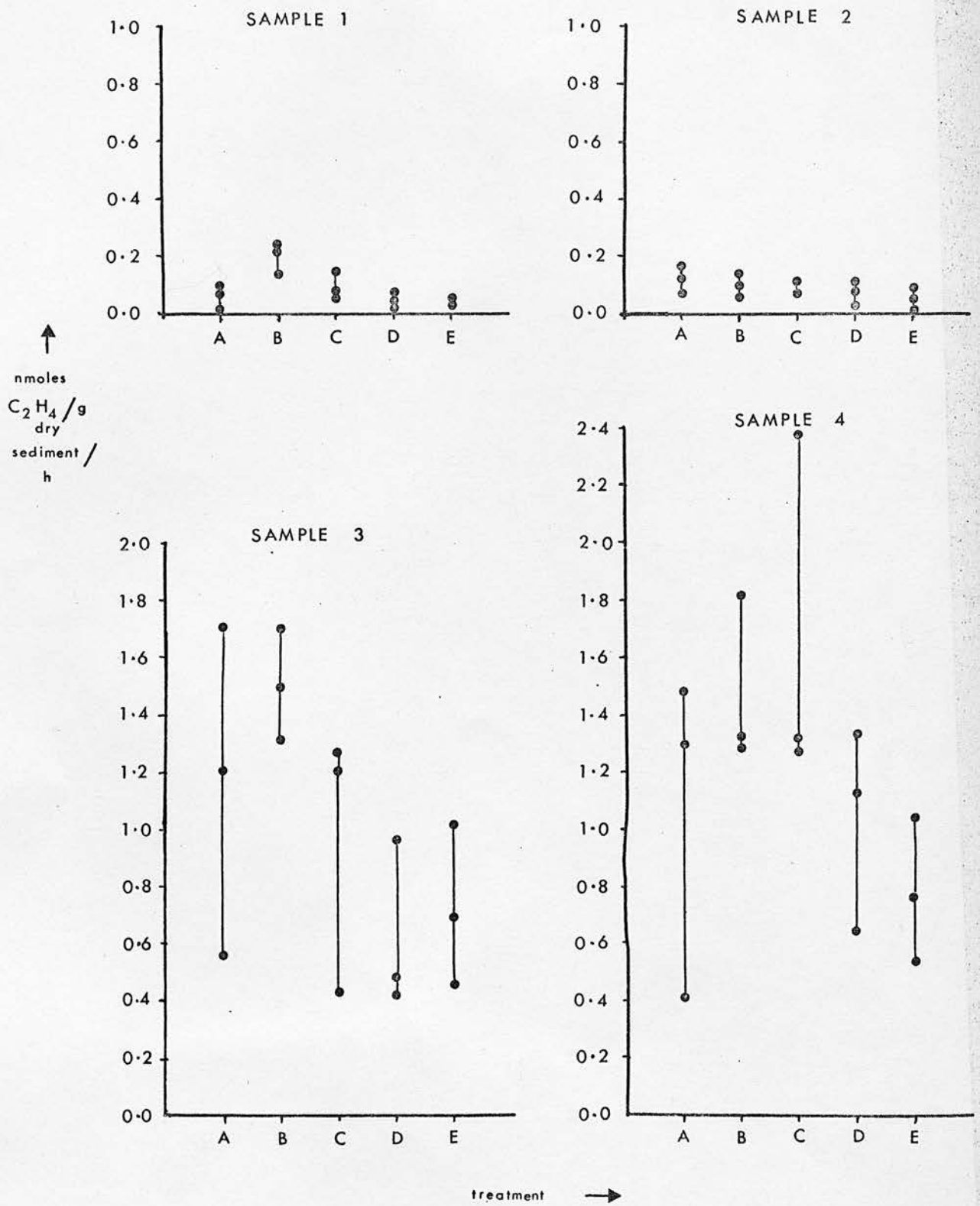


FIG 3-3

(EXPERIMENT 2)

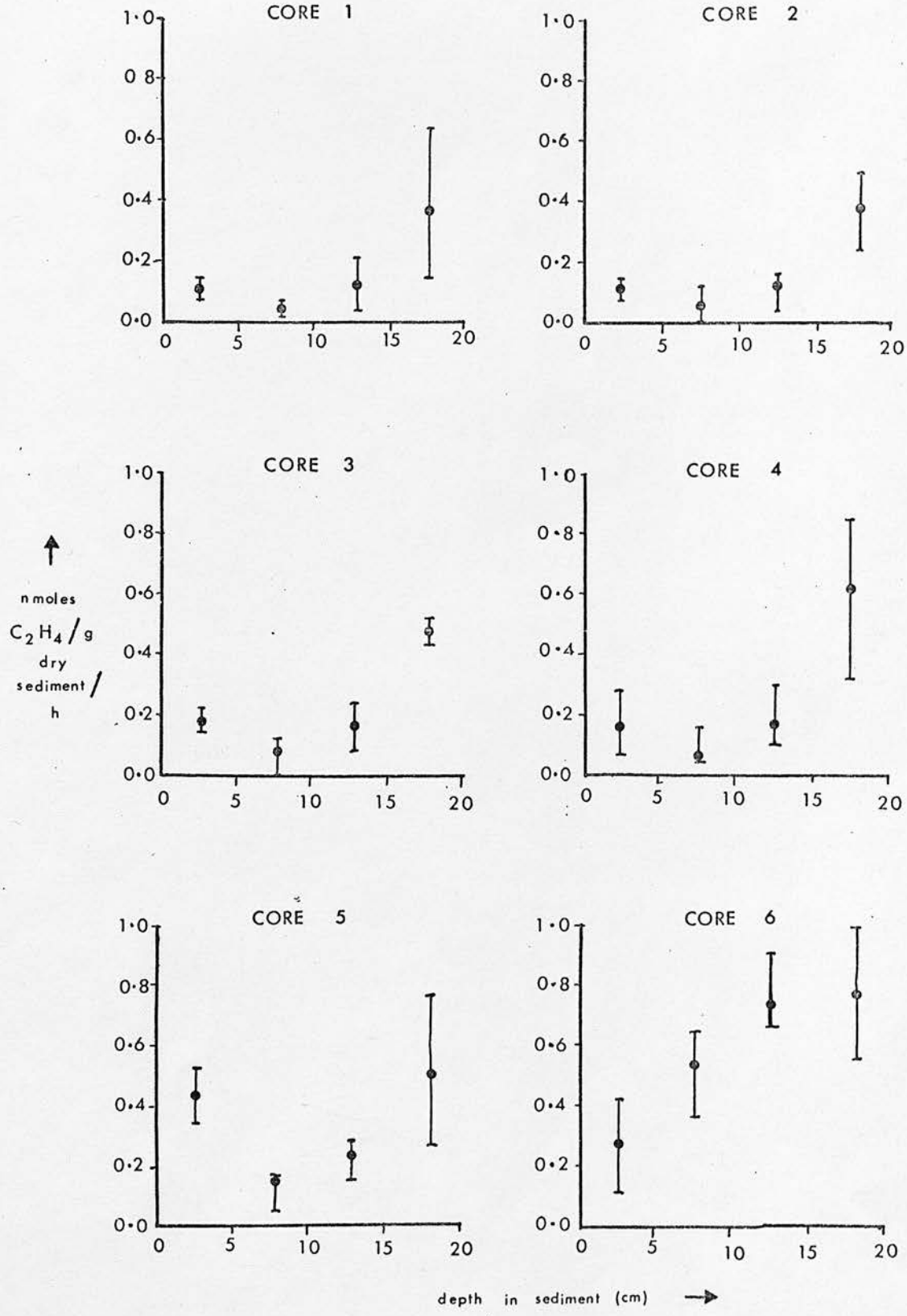
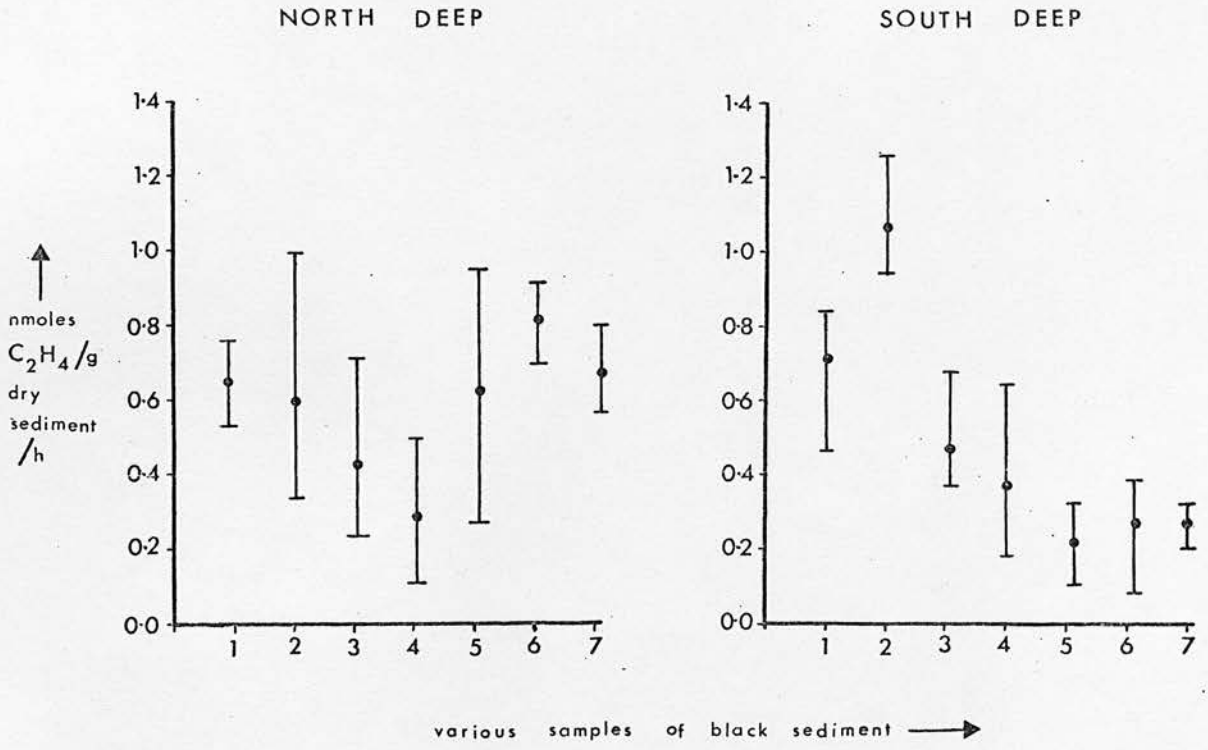


FIG 3-4

(EXPERIMENT 3)



TREATMENTS USED IN FIG. 3-2

Conditions of Incubation / Treatment	light or dark	atm. O_2 in gas phase	temperature (^{o}C)
A	light	0.00	23
B	dark	0.00	25
C	dark	0.05	25
D	dark	0.10	25
E	dark	0.20	25

OTHER EXPERIMENTAL CONDITIONS

Fig. no. / Conditions	Fig. 3-2	Fig. 3-3	Fig. 3-4
Sample collection date	5/2/1974	13/3/1974	19/6/1974
Loch temperature (^{o}C)	4	5	15
Incubation temperature (^{o}C)	see above	25	15
Flushing gas	OfN_2	OfN_2	OfN_2
Atm O_2 in gas phase	see above	0.2	0.0
Atm C_2H_2 in gas phase	0.2	0.1	0.1
Preincubation	overnight	overnight	none
Incubation with C_2H_2 (h)	24	12-17	15

Figures 3-2, 3-3 & 3-4

Ethylene production in acetylene reduction assays of freshly sampled sediment from various sites in Loch Leven (location of sites is shown on map, Figure 3-1).

Figure 3-2 (Experiment 1)

Ethylene produced by triplicate samples of sediment under different conditions (A-E, see table) from Ekman Grab samples collected at four 333 m intervals along the transect line, and numbered 1-4 towards North Deep, sample 4 being from the bottom of North Deep. Points (●) are individual values.

Figure 3-3 (Experiment 2)

Average (●) of ethylene produced by triplicate samples of sediment from four 5 cm deep slices of each of four Jenkins cores collected at six 200 m intervals along the transect line, core one being from the shallow end and core six from North Deep. Range (highest and lowest values) is also shown (⊢——⊣).

Figure 3-4 (Experiment 3)

Average (●) of ethylene produced by four replicate samples of various samples of black sediment from four from cores each of North and South Deeps. Range (highest and lowest values) is also shown (⊢——⊣).

FIG 3-5

(EXPERIMENT 4)

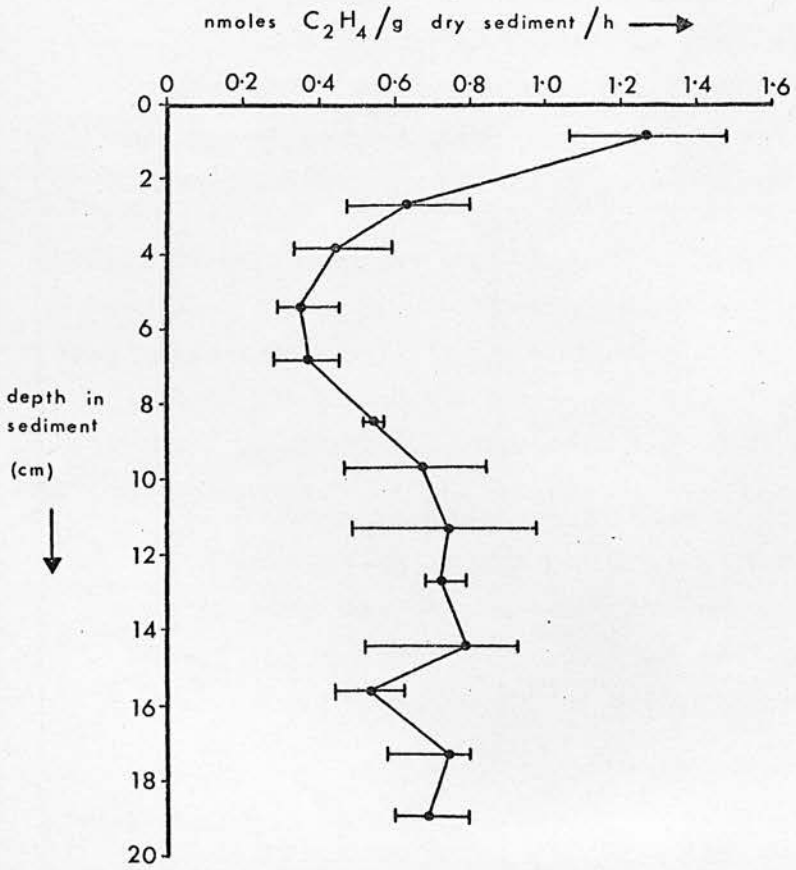


Figure 3-5 (Experiment 4)

Average of ethylene produced in acetylene reduction assays of triplicate samples from 15 x $1\frac{1}{2}$ cm thick slices of one Loch Leven North Deep Jenkin's core collected on 10th July 1974. Range (⊢——⊣) also shown.
Conditions: lake temperature 15°C; incubation temperature 15°C; anaerobic gas phase (OfN_2); 25.5 hour incubation with 0.1 atm C_2H_2; no pre-incubation.

the sediment from South Deep, although mainly black, was otherwise different in appearance from North Deep sediment, in that it contained a large amount of undecomposed organic matter and was more solid in consistency.

In Experiment 4 it was observed that there was a layer of black sediment both at the top and the bottom of the core samples, and it appeared that this was not due to up-welling of black sediment from beneath, as occurred in Experiment 2 because there were no channels through the brown sediment. It was concluded that this reflected a seasonal change in the sediment, a black layer being formed on the surface of the sediment in North and South Deeps during the summer, and disappearing in the autumn. Inspection of samples collected at other times of the year, both by myself and other workers, confirmed this conclusion.

Examination of Figures 3-2 to 3-5 reveals that the differences in appearance of the samples relate closely to the rates at which ethylene was produced in the acetylene reduction assays. In Experiments 1 and 2 black sediment always produced more ethylene. In Experiment 3 ethylene production from South Deep sediment was more variable than that from North Deep sediment, which might have been expected considering its more uneven consistency. In Experiment 4, the change in rate of ethylene production with depth in the 1.5 cm thick slices of the core correlates closely with the changes in sediment colour observed.

The different treatments applied in Experiment 1 also affected ethylene production; in Figure 3-2 it can be seen that higher levels of oxygen in the gas phase inhibited ethylene production. Light did not increase ethylene production, and although this does not definitely show that blue-green algae are not responsible (see section 3-1), it does seem unlikely that they would flourish below the surface of the sediment.

It may probably be assumed that the distinctness between black and brown lake sediment is due to a predominance of ferric salts in the brown sediment and ferrous sulphide in the black, because black sediment is more reduced (many other workers have distinguished between black and brown sediment e.g. Mortimer, 1941 & 1942). The observation that methane production is greater from black than brown sediment confirms this assumption, and indicates that at least parts of the sediment are completely anaerobic. Thus the greater ethylene production from black than brown sediment and its inhibition by oxygen implies that organisms

which fix more nitrogen under anaerobic conditions are responsible.

Conclusion. The results distinguished two types of sediment in Loch Leven; black and brown. The distribution of these types varied with depth within the loch and within the sediment itself. It also seemed to change seasonally. The rate of ethylene production in acetylene reduction assays correlated closely with the blackness of the sediment, and it seems most likely that it was caused by anaerobic nitrogen fixers, as it was inhibited by oxygen. Thus, although there was not a direct correlation between the organic carbon content of the sediment and with ethylene production rates, as would be expected, organic carbon content may be indirectly involved by being related to water depth and the blackness of sediment. The possibility of nitrogen fixation at the sediment surface in the summer is of particular interest because the nitrogen fixed would be in a better position to be transferred to the lake water than that fixed deeper in the sediment. Also, an anaerobic layer at the sediment surface would probably encourage sediment water transfer of nitrogen compounds (Mortimer, 1941 & 1942). Thus it would be of value to the understanding of nitrogen cycling in Loch Leven to investigate the extent and effects of anaerobiosis in the sediment, particularly in the surface layer.

The effect of temperature is discussed later (section 3-6).

3-4 Experiments 5 and 6: Attempts to determine the organisms responsible for nitrogen fixation in Loch Leven sediment and to show the statistical reliability of differences in rates of ethylene production in acetylene reduction assays.

If a correlation can be shown between numbers of nitrogen fixing organisms and nitrogenase activity in a sample, it is implied that the organisms being counted are responsible for the nitrogenase activity. In Experiments 5 and 6 a counting method, selective for vegetative and non-vegetative endospore forming nitrogen fixers, was used. The numbers were compared with the results of acetylene reduction assays in black and brown sediment (Experiment 5) and in black aerated and non-aerated sediment (Experiment 6), as it seemed from the results of section 3-3 that anaerobic nitrogen fixers might be active in the sediment. Such counting

methods are necessarily selective; they do not include all species of nitrogen fixers. Some enrichments for other species were therefore included simply to determine their presence or absence in the sediment. Previous attempts to enrich methane oxidizers from Loch Leven sediment had been successful (Dr. E. Williams pers. comm.). They were not therefore repeated here.

In Experiment 5 statistical analysis was carried out to ensure that the differences observed between ethylene production by black and brown sediment under aerobic and anaerobic conditions were significant.

Materials and Methods.

Experiment 5. Four Jenkins cores were collected on 16th April 1974 from Loch Leven North Deep (lake temperature 7°C). In these samples the black sediment did not well up through cracks in the brown as in Experiment 2, and there were only two layers of sediment, a layer of brown sediment overlying a layer of black, each of approximately equal thickness. In the laboratory the sediment in each core was separated according to its colour (black or brown), and eight approximately 3 ml amounts of each part were distributed into pre-weighed bottles. Semi-aerobic and anaerobic conditions were established over four replicates of each part by adding 2 ml O_2 to half the bottles after they had all been made anaerobic. 2 ml acetylene were then added to each bottle and they were incubated at 7°C for 15-18 hours, when the gas phase was analysed for ethylene. The results (nmoles ethylene per gram dry weight per hour) were analysed statistically by analysis of variance, and Duncan's multiple range test.

The most probable number counting technique, was adapted from Campbell & Evans (1969). Four samples each of black and brown sediment from one core were added to 9 ml amounts of phosphate buffered saline (pH 7.0) containing 1.0% Calgon (Na hexametaphosphate) and shaken for ten minutes on a wrist action shaker. The wet weight of sediment added to give these 10^{-1} dilutions was determined by weighing the bottles before and after addition of the sediment. Separate weighed amounts were dried to determine the per cent dry weight of the sediment. One of the duplicate sets of four 10^{-1} dilutions was pasteurized at 80°C for ten minutes. 10^{-2}, 10^{-3} and 10^{-4} dilutions were made from each of the eight 10^{-1} dilutions by transferring 1 ml along a series of 9 ml amounts of the medium described below in stoppered bottles which had been flushed out

with OfN_2. 1 ml samples from each dilution were injected into eight replicate sealed sterile anaerobic 1 oz bottles containing 10 ml of the following medium: 1000 ml distilled water; 0.2 g $MgSO_4.7H_2O$; 0.01 g $FeCl_3$; 0.002 g $NaMoO_4.2H_2O$; 0.02 g $CaCl_2$; 0.1 g $NaHCO_3$; 10 g sucrose, to which 0.472 g/1 KH_2PO_4, 0.874 g/1 K_2HPO_4 and 0.1% w/v yeast extract were added after autoclaving (final pH 7.2). The 256 bottles were incubated for three days at 30°C after which 2 ml acetylene were added to each. After another 24 hours at the same temperature the gas phase was analysed for the presence of ethylene. The most probable number of nitrogen fixing organisms was calculated from the number of bottles in each set of eight which had reduced acetylene, according to the table compiled by Harris & Sommers (1968). The organisms in some of the positive bottles were examined microscopically.

Enrichment cultures for Azotobacter and Desulfovibrio were set up as follows: For Azotobacter, 5 mg mannitol/ml sediment was added to each of six approximately 50 ml samples of sediment, which were then placed in flasks stoppered with cotton wool bungs on a shaker at 30°C. After three days the sediment was streaked onto nitrogen free mannitol agar plates (medium as above, substituting mannitol for sucrose). For Desulfovibrio, three 50 ml samples of sediment were placed in 250 ml conical "Quickfit" flasks, and 6.3 mg Na lactate and 1 mg $FeSO_4$/ml sediment were added. The flasks were stoppered with Suba-seals, flushed with OfN_2, and incubated statically at 30°C. After three days the sediment was tested for its ability to reduce acetylene and examined under a microscope.

Experiment 6. On 29th May 1974 four Jenkins cores were collected from Loch Leven North Deep. The black sediment was separated from each core, diluted with OfN_2-flushed lake water, mixed together, and divided between three conical flasks with cotton wool bungs. A sample from each flask was removed for a count and the remaining sediment was shaken in a water bath at lake temperature (15°C) for 1-5 days to aerate it, after which the count was repeated. The counting method was fundamentally the same as that described above (Experiment 5) except that the Calgon was reduced to 0.5% as recommended by Babiuk & Paul (1970); shaking on the wrist action shaker was increased to 15 minutes; the number of dilutions inoculated was increased to 10^{-3} to 10^{-7} for pasteurized and 10^{-3} to 10^{-8} for non-pasteurized sediment; 0.5 g Na thioglycollate was included in the medium.

TABLE 3-1 (Experiments 5 and 6). Most probable number (MPN) counts of endospore-forming nitrogen fixers in dilutions of black, brown, and initially black aerated and non-aerated sediment, collected from Loch Leven North Deep on 16.4.1974 and 29.5.1974.

Experiment 5: average MPN of duplicate samples

		MPN / g dry sediment
black sediment	pasteurized	68,333
	non-pasteurized	≥ 151,754
brown sediment	pasteurized	23,421
	non pasteurized	≥ 151,754

Experiment 6: average MPN of triplicate samples (initially black sediment)

		MPN / g dry sediment
before aeration	pasteurized	21,052
	non-pasteurized	177,643
after aeration	pasteurized	2,672
	non-pasteurized	96,915

TABLE 3-2 (Experiment 5). Nanomoles C_2H_4 produced / g dry weight individual sediment sample / h. Loch Leven North Deep sediment collected on 16.4.1974.

Treatment / Core no.	A	B	C	D
I	.0182 0 .0138 0	.0790 .1003 .0958 .0228	.1746 .1790 .1745 .1841	.3252 .2380 .3928 .3459
II	.0193 0 0 0	.0188 0 .1464 .0940	.1349 .1541 .2132 .2722	.3332 .4207 .3091 .2523
III	.0773 .0027 0 .0626	.1701 .2175 .0812 .1598	.1911 .2418 .1952 .1791	.3523 .3848 .3758 .2883
IV	.0571 .0571 .0683 .0503	.0875 .0568 .0434 .0452	.1855 .1441 .1459 .1429	.3332 .2641 .2931 .2820
Treatment means	.0263	.0887	.1820	.3244

Treatments:

A Brown sediment; aerobic incubation
B Brown sediment; anaerobic incubation
C Black sediment; aerobic incubation
D Black sediment; anaerobic incubation

ANALYSIS OF VARIANCE

	Sums of Squares	d.f.	Mean Square	F
Treatment	0.8063	3	0.2688	192*
Core	0.0208	3	0.0070	5+
Interaction	0.0156	9	0.0017	1.2143 n.s.
Total	0.9046	63		
Residual	0.0619	45	0.0014(s^2)	

* significant at all levels
\+ significant at 5%
n.s. not significant

$D^{1\%}$(Duncan's factor)= .0008

The sediment was also tested before and after aeration to show whether it reduced acetylene.

Results and Discussion. The results of both the counts are shown in Table 3-1 and the analysis of the rates of ethylene production in Experiment 5 in Table 3-2. It was found by microscopic examination that nearly all the organisms which grew in the counting media were Gram-positive endospore forming rods, which are assumed to have been nitrogen fixing Bacillus or Clostridium species. These organisms might have been present in the sediment as endospores, and as such inert. However, the difference in numbers between pasteurized and non-pasteurized treatments (Table 3-1) indicates that at least some of them were present in the vegetative form. In Experiment 6, both pasteurized and non-pasteurized treatments gave higher results in the non-aerated than in the aerated sediment. In Experiment 5 all the dilutions of both black and brown non-pasteurized sediment were positive. Therefore if the numbers had been higher in black than brown sediment it would not have been detected. It seems likely that this was the case, as the numbers in the pasteurized black sediment were higher than in the pasteurized brown. Thus it seems that the count did reveal a difference in numbers between the black and brown sediment. The acetylene reduction assays in Experiment 6 showed that the sediment reduced much more acetylene before than after aeration. The results of the acetylene reduction assays in Experiment 5 are shown in Table 3-2, where it can be seen that the differences between cores were significant at the 5% but not 1% probability level, whereas the difference between treatments was highly significant. This confirms the conclusions of section 3-3, that black sediment produces more ethylene than brown, and that ethylene production is inhibited by oxygen, and also shows that the numbers of nitrogen fixing organisms counted correlate with the rates of ethylene production by black and brown sediment. This implies that the organisms being counted (Clostridium and/or Bacillus spp) are responsible for the ethylene production. Presumably the lower rate of ethylene production by the brown sediment is due to inhibition of the nitrogen fixing activity of these organisms by more aerobic conditions. This is supported by the conclusion of Keeney (1973), that Clostridium spp are most likely to cause sediment nitrogen fixation. However, the enrichment cultures set up for other types of nitrogen fixers in

Experiment 5 were also successful: a large number of pale brown mucoid colonies containing Azotobacter-like cells were formed on the mannitol agar plates from the Azotobacter enrichments, and the sediment enriched with lactate and sulphate contained a predominance of Gram-negatively-staining curved rods resembling Desulfovibrio, which reduced acetylene vigorously. It is therefore possible that the ethylene production in fact reflects the activity of other species of nitrogen fixer in addition to Clostridium and/or Bacillus species. For example, the activities of Clostridium and Desulfovibrio would not have been distinguished by the treatments used in the acetylene reduction assays.

Conclusion. It was found that the numbers of nitrogen fixing Clostridium and/or Bacillus spp. correlated with rates of ethylene production in acetylene reduction assays, but that other species of nitrogen fixing organisms were also present (probably including methane oxidizers). Therefore, even if Bacillus and/or Clostridium spp. are responsible for nitrogen fixation in Loch Leven sediment, a change in conditions could well result in a different species of nitrogen fixer becoming dominant. If different species of nitrogen fixers become dominant in different sediment types, estimates of the total quantity of nitrogen being fixed will be affected, because the conditions of the assay may differentially stimulate or inhibit the various species. Also, if conditions change in the loch, the rate of nitrogen fixation will be affected differently depending on the dominant species. Therefore detailed investigations of nitrogen fixation in Loch Leven North Deep were discontinued, so that samples from other lakes likely to have different conditions and populations in their sediments could be collected and tested.

3-5 Experiments 7 to 10: Comparison of rates of ethylene production in acetylene reduction assays of Esthwaite and Grasmere sediments at different depths in the sediment and under aerobic and anaerobic conditions of incubation.

Magdoff and Bouldin (1970) showed, that in waterlogged soil systems, Azotobacter can be very active where aerobic and anaerobic conditions are in close proximity, and Barrow and Jenkinson (1962) found higher rates of nitrogen fixation in waterlogged soil under aerobic conditions. Thus it is possible that aerobic nitrogen fixers might be

important in certain types of lake sediment.

Esthwaite and Grasmere, two eutrophic lakes in the English Lake District, undergo temperature stratification for 4-5 months in the summer. On the other hand, in Loch Leven stratification only occurs for short intermittent periods in the North and South Deeps, because the main part of the loch is shallow and exposed to wind (Smith, 1974). The phenomenon of temperature stratification in lakes is well documented (e.g. Hutchinson, 1957); dense cold water becomes separated from lighter warmer water by a "thermocline". In Esthwaite and Grasmere the lower layer of water (the "hypolimnion"), which is separated from the atmosphere by the thermocline, becomes anaerobic owing to respiratory oxygen uptake. This only seldom occurs in Loch Leven. Such differences in the dissolved oxygen and turbulence of the water overlying the sediment are likely to affect sediment Eh. Therefore it seemed that these differences might affect the nitrogen fixing population in the sediments.

Experiments 7-10 were carried out to show whether aerobic nitrogen fixers could be active in Esthwaite and Grasmere sediment, since they were apparently inactive in Loch Leven.

It should be possible to demonstrate the activity of different groups of nitrogen fixers in the sediment by means of applying the acetylene reduction assay under different sets of conditions. However, if more than one species of nitrogen fixer is present and active in one sediment, their response in acetylene reduction assays to different treatments will be that of the predominant species. Thus even if aerobic nitrogen fixers were active in the sediments of Esthwaite and Grasmere, their activity would only be detected by this method if it predominated over that of anaerobes, under the experimental conditions used. The conditions chosen were "anaerobic" and 0.05 atm O_2 ("semi-aerobic") at 10°C.

Methods and Materials. Experiment 7. Four Jenkins cores were collected from the deepest point (15 m) in Esthwaite Water on 25th June 1974 (core temperature 8.5°C; dissolved O_2 2%). There were small particles of red-brown sediment on the surface of the cores, but apart from this the sediment was completely black. In the laboratory at Ferry House, Windermere, the water was siphoned off the cores and the sediment was sliced into fifteen 1 cm thick slices from the surface downwards using a piece of equipment especially designed for the purpose.

Two samples (approximately 3 ml) from each slice were transferred into pre-weighed bottles. The bottles were sealed, evacuated, and flushed with argon. 0.05 atm O_2 was added to one bottle of each pair. They were incubated with 10% acetylene for 17 hours at 10°C. A preliminary experiment was carried out using 2 ml 50% aqueous solution of trichloroacetic acid per bottle to stop the reaction by protein precipitation, so that the bottles could be transported back to Edinburgh for analysis of the gas phase. However, acetylene reduction did not stop immediately. Thus, instead, samples of the gas phase were taken into 1 ml disposable plastic syringes fitted with needles which were closed by pushing them into rubber bungs fitted into test tube racks. In order to avoid the risk of leakage of the gas samples during the journey, three samples were taken from each bottle. They were analysed in Edinburgh the same day. It was found that the syringes seldom leaked.

Experiment 8. Three Jenkins cores were collected from Grasmere at the deepest point (20 m) on 5th July 1974 (core temperature 7°C). The surface of the sediment was black. Ten 1 cm thick slices of each core were made, and ethylene production by triplicate samples from each slice was determined after 14 hour incubation under semi-aerobic and anaerobic conditions at 10°C, as in Experiment 7.

Experiment 9. Two Jenkins cores were collected from the deepest point in Grasmere on 26th August 1974, when the temperature in the cores was 5°C. Fifteen 1 cm thick slices were made from each core and ethylene production by four replicate samples from each slice under both semi-aerobic and anaerobic conditions at 10°C was determined as in Experiment 7, after 13 hours incubation.

Experiment 10. Two Jenkins cores were collected from the deepest point in each of Esthwaite Water and Grasmere on 4th November 1974. Lake temperature was 10°C and the water column in both lakes was uniformly saturated with oxygen. Surface layers of all the cores were predominately black but contained particles of red-brown material. The sediment was sliced into seven 1 cm thick slices at 1, 2, 3, 6, 9, 12 and 15 cm depth. Ethylene production by four replicate samples of each slice under both semi-aerobic and anaerobic conditions was measured as in Experiment 7,

FIG 3-6

(EXPERIMENT 7)

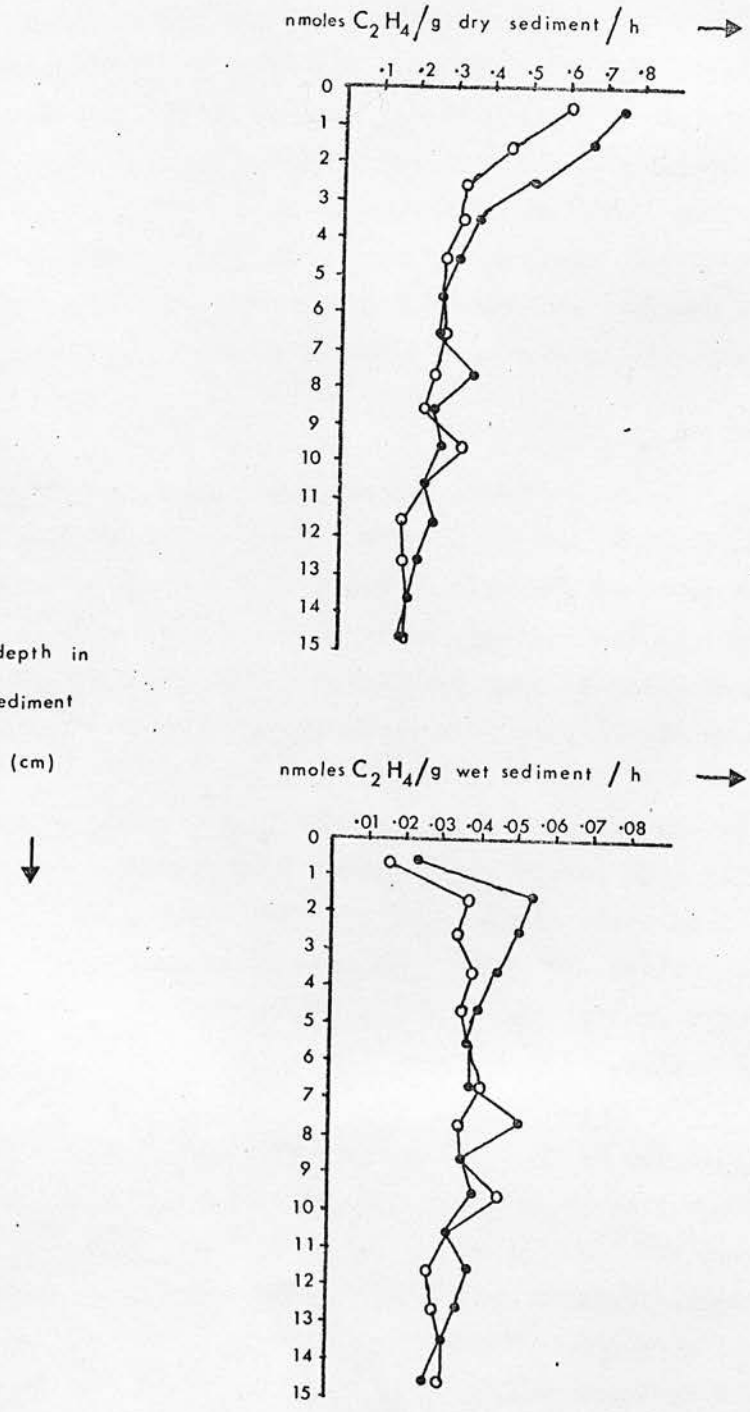


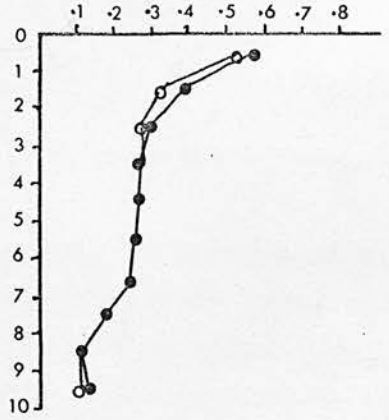
Figure 3-6 (Experiment 7)

Average of ethylene produced in acetylene reduction assays of single samples from 1 cm thick slices of four Jenkins Cores collected from Esthwaite Water on 25th June 1974. Conditions: core temperature 8.5°C; incubation temperature 10°C; 0.05 (○) or 0.00 (●) atm O_2; flushing gas Ar; 17 hours incubation with 0.1 atm C_2H_2; no pre-incubation. Results expressed per gram dry and wet weight.

FIG 3-7

(EXPERIMENT 8)

nmoles C_2H_4 / g dry sediment / h →



depth in sediment (cm) ↓

nmoles C_2H_4 / g wet sediment / h →

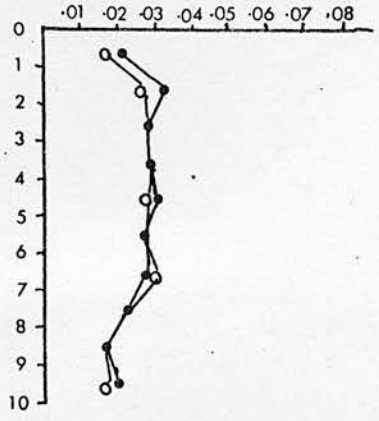


Figure 3-7 (Experiment 8)

Average of ethylene produced in acetylene reduction assays of triplicate samples from 1 cm thick slices of three Jenkins cores collected from Grasmere on 15th July 1974. Conditions: core temperature 7°C; incubation temperature 10°C; 0.05 (o) or 0.00 (●) atm O_2; flushing gas Ar; 14 hours incubation with 0.1 atm C_2H_2; no pre-incubation. Results expressed per gram dry and wet weight.

FIG 3 - 8

(EXPERIMENT 10)

ESTHWAITE

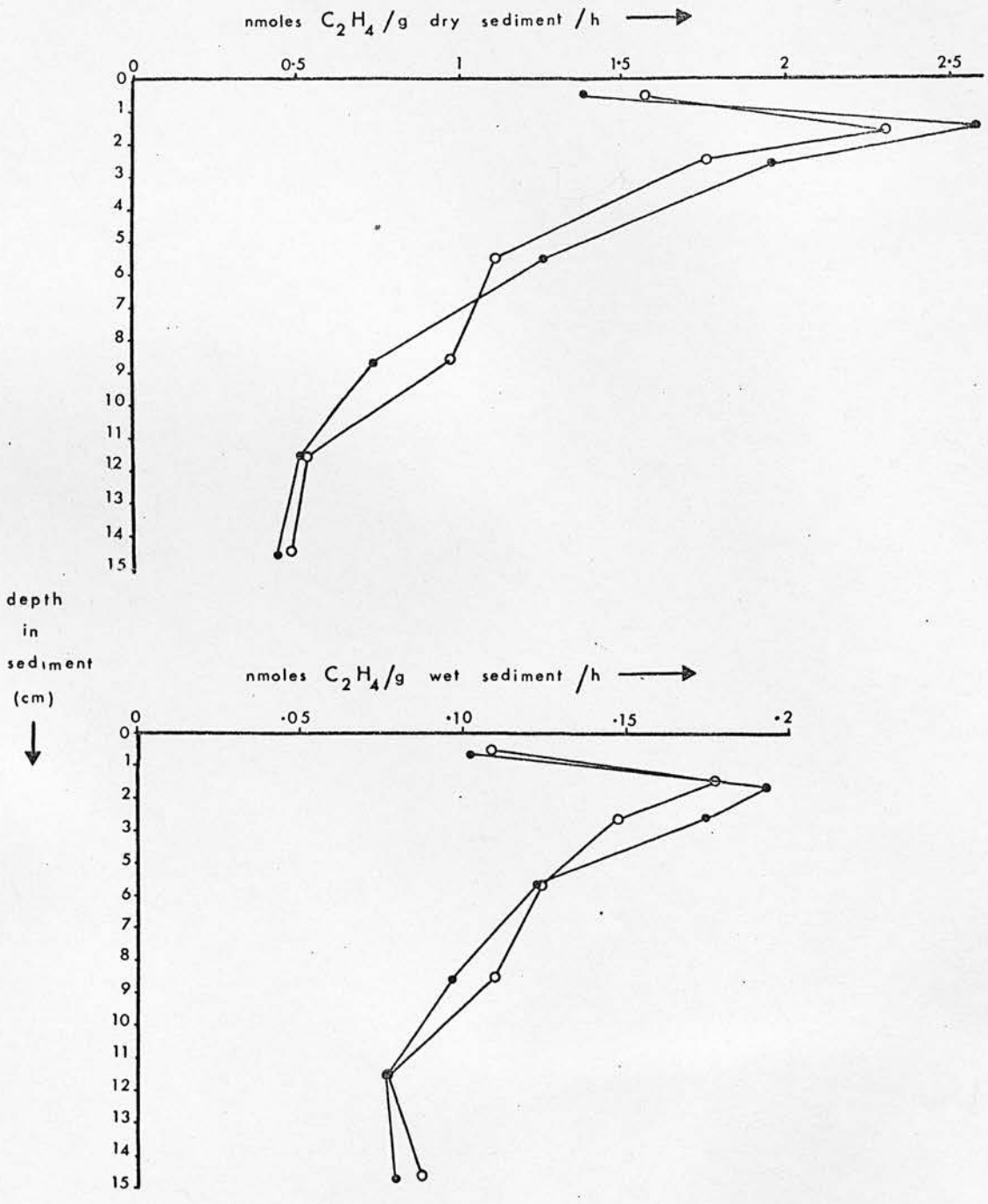


FIG 8 (cont.)

(EXPERIMENT 10)

GRASMERE

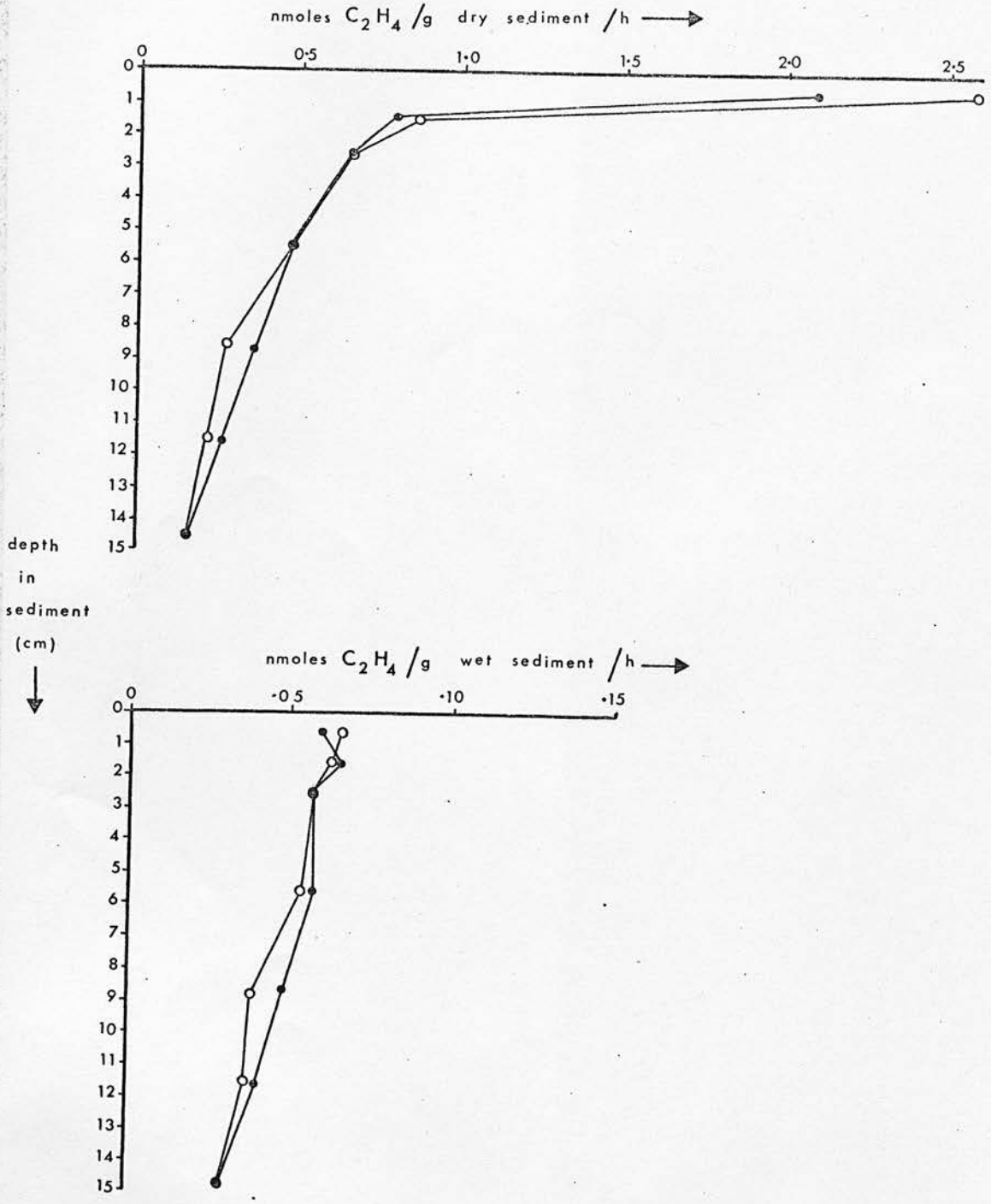


Figure 3-8 (Experiment 10)

Average of ethylene produced in acetylene reduction assays of four replicate samples from seven 1 cm thick slices of two Jenkins cores collected from each of Esthwaite Water and Grasmere on 4th November 1974.
Conditions: core temperature 10°C; incubation temperature 10°C; 0.05 (O) or 0.00 (●) atm O_2; flushing gas Ar; 12-17 hours incubation with 0.1 atm C_2H_2; no pre-incubation. Results expressed per gram dry and wet weight.

after 12-17 hours incubation.

Results and Discussion. The results are shown in Figures 3-6 to 3-9. They have been calculated in terms of both wet and dry weight of sediment. Most published results of acetylene reduction assays on lake sediments are expressed per gram dry weight. However, the proportion of dry matter in the sediment increases with depth and this could give the impression that activity decreases with depth, when no such change is observed if the results are expressed on a wet weight or volume basis. This effect was observed in some of the experiments (e.g. Experiment 8 Figure 3-7). The water contained in the sediment in the natural habitat should be considered to be part of the sediment, rather than a factor which dilutes sediment activity; wet weight or volume based measurements therefore have some advantages over a dry weight basis if the activity of different sediments is to be compared. However, the surface few millimetres of the sediment is probably a very important area of sediment activity (e.g. Reuszner, 1933; Henrici & McCoy, 1938). When it is being sampled this zone is much more susceptible to dilution by the overlying water than is the rest of the sediment which might explain why other workers (e.g. Hayes & Anthony, 1959) were unable to demonstrate it. Ethylene production in this zone would also be severely under-estimated if it was expressed on a volume or wet weight basis. Thus, in order to assess the activity of these few surface millimetres more accurately, it would be necessary to devise a sampling technique which avoided the disturbance of the sediment.

It can be seen in Figures 3-6 to 3-8 that the results of Experiments 7-9 were considerably lower than those of Experiment 10. No ethylene was produced at all in Experiment 9. The samples in Experiments 7-9 were taken during the summer stratification period. On the other hand, in mid-October windy weather and lower temperatures break up the stratification, a process known as the "overturn", and the samples in Experiment 10 were taken two weeks after this. A small increase in temperature at the sediment surface does occur at the overturn ($2^{o}C$), and it is possible that this caused the increase in ethylene production which occurred in Experiment 10. However, the effect of temperature on ethylene production by Loch Leven sediment was not this great (see section 3-6). It seems that the ethylene production in Experiment 10

FIG 3-9

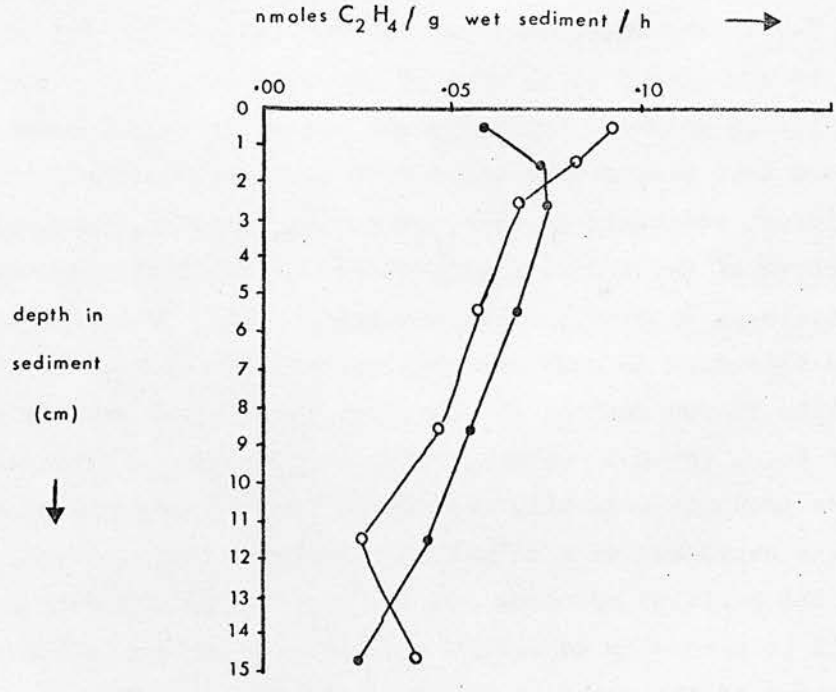

nmoles C_2H_4 / g wet sediment / h
·00
·05
·10
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
depth in
sediment
(cm)

Figure 3-9 (Experiment 10)

Average ethylene produced in acetylene reduction assays of four replicate samples of seven 1 cm thick slices of a single Jenkins core collected from Grasmere on 4th November 1974, showing differential effect of semi-aerobic (0.05 atm O_2; O) and anaerobic (0.00 atm O_2; ●) conditions.
Conditions as specified in legend to Figure 3-8.

was due to the activities of aerobic nitrogen fixers: at the overturn conditions at the sediment surface suddenly become aerobic. Organic carbon, which presumably accumulates during the summer stratification period owing to the lower efficiency of anaerobic processes, is thus made available for aerobic respiration. The activity at the surface of individual cores was sometimes higher under aerobic than anaerobic conditions (Figure 3-9), which implies that aerobes are more active at the surface, and anaerobes at greater depth. However, on average, there was not a very pronounced difference between the two treatments. This could be because the activity of aerobic nitrogen fixers was disguised by that of anaerobes, but an alternative or additional explanation could be that increased aerobic decomposition at the overturn makes more carbon available for the activities of anaerobic nitrogen fixers. Whatever the reason for the increase, it appears that in these stratifying lakes sediment nitrogen fixation may be important in the period which follows the overturn, and this possibility would merit further investigation.

Conclusion. Jenkins cores collected from Esthwaite Water and Grasmere showed much higher rates of ethylene production in acetylene reduction assays after the autumn overturn than during the summer stratification period. It is possible that this was due to aerobic nitrogen fixers. Even if it were due to anaerobes it is clear that the lake conditions which affect the rates of ethylene production are different in Esthwaite and Grasmere than in Loch Leven. This difference between stratifying and non-stratifying lakes should be taken into account when considering the total quantity of nitrogen being fixed in lake sediments. Further experiments could be designed to show whether the phenomenon observed was repeated each autumn, and which organisms were responsible.

3-6 Effect of temperatures on ethylene production in acetylene reduction assays of lake sediments. Howard et al (1970) concluded that, in their sediment samples, temperatures between 3° and 23°C had no effect on acetylene reduction rates. However, it was found in section 2-2 (Figure 2-1) that incubation temperature did affect ethylene production rates in acetylene reduction assays of Loch Leven sediment. Thus it

seems likely that environmental temperatures would also affect it. The temperature at the sediment surface of Esthwaite Water and Grasmere seldom rises above 8°C because these lakes undergo temperature stratification. On the other hand, in Loch Leven the whole water column varies between 4°C in the winter and even 30°C in the summer, and the surface sediment temperatures presumably change similarly. Examination of some of the results already described reveals that they do reflect these temperature differences. For example, in Experiments 4 and 5, which were carried out at 15°C and 7°C (lake temperature) respectively, more ethylene was produced at the higher temperature (c.f. Figure 3-5 and Table 3-2). Also, the Lake District samples produced much less ethylene during the summer stratification period than the black Loch Leven sediment in Experiment 4, which was collected at the same time. The sediment of Esthwaite and Grasmere must have been completely anaerobic during this period and thus if it is not affected by temperature, ethylene production might be expected to be the same as in black Loch Leven sediment. If temperature does indeed affect nitrogen fixation in lake sediments, it is obviously important to take this into account when measuring the amount of nitrogen being fixed.

3-7 Summary of main conclusions, and suggestions for further work.

In Loch Leven the highest rates of ethylene production in acetylene reduction assays were observed in black sediment, which occurred mainly in the North and South Deeps, more extensively in the summer than the winter. Thus the North and South Deeps may play a special role in nitrogen cycling in Loch Leven. They form only a small proportion of the total expanse of sediment, however, so in future studies of nitrogen cycling, their importance relative to brown sediment would have to be assessed. The ethylene producing activity of the sediment was inhibited by oxygen, and experiments showed that changes in numbers of certain nitrogen fixing bacteria likely to be *Clostridium* or *Bacillus* spp. correlated with changes in the rate of ethylene production. However, tests for the presence of other nitrogen fixing bacteria (*Azotobacter* and *Desulfovibrio*) were also positive, and other nitrogen fixers not tested for might also be present. Thus in a different habitat one of

these other species might become dominant. Indeed, high rates of ethylene production were observed in the sediments of Esthwaite and Grasmere two weeks after the overturn, and there were indications that this might be due to a different species of nitrogen fixer than the ethylene production in Loch Leven.

Higher rates of ethylene production by Loch Leven sediment occurred in summer than in winter, and it was also found that the rates of ethylene production by Esthwaite and Grasmere sediment during the summer stratification period were lower than in Loch Leven at the same time. It is likely that this was due to differences in the environmental temperature, even though Howard et al (1970) obtained the opposite result. Thus it seems that temperature should be taken into account when attempting to measure sediment nitrogen fixation.

To investigate the ecology of nitrogen fixation in these sediments further, it would be necessary to measure sediment Eh, rather than to rely on the colour of the sediment. Other factors, such as the carbon and nitrogen levels, could also be investigated, both by direct measurement, and by adding carbon and nitrogen to the sediment. Counts of a wider variety of nitrogen fixers could be carried out, both in Loch Leven North Deep and in Esthwaite and Grasmere after the overturn. It would also be important to investigate the role of nitrogen fixing methane oxidizers, as their activity would not have been detected by the methods used here. They might be important either in Loch Leven (e.g. Loch Leven brown sediment) or Esthwaite and Grasmere, or both lake types. An alternative method, such as $^{15}N_2$-enrichment from $^{15}N_2$ would also be needed to complement the results of the acetylene reduction assays, and the acetylene reduction assays might be improved by diluting and shaking the sediment, as suggested in Chapter 2.

CHAPTER 4. ASYMBIOTIC NITROGEN FIXATION IN THE RHIZOSPERE OF AQUATIC MACROPHYTES IN LOCH LEVEN (KINROSS).

4-1 Introduction

Experiments in which cultures of nitrogen fixing bacteria from the roots of the aquatic macrophyte Potamogeton filiformis were investigated are described in a separate pre-print (see back of thesis). The work described in this chapter consists of the acetylene reduction assays various samples including roots which supported the bacteriological investigations, and a more detailed discussion of the literature relating to rhizosphere nitrogen fixation.

It is now known that nitrogen fixation by asymbiotic bacteria in the rhizosphere of certain terrestrial plants can provide a significant proportion of a plant's nitrogen requirement (e.g. Von Bulow & Dobereiner, 1975). Recent studies have shown that asymbiotic nitrogen fixation may also occur in the rhizosphere of aquatic macrophytes. Bristow (1974) found significant rates of acetylene reduction in the roots of freshwater angiosperms, and Patriquin & Knowles (1972) of both ^{15}N-enrichment from $^{15}N_2$ and acetylene reduction in marine macrophytes. Zuberer & Silver (in press) studied nitrogen fixation in the rhizosphere of mangroves, and deduced that the process might be a critical factor in the establishment of mangrove swamps. Although a causal relationship was not established, these workers found that there were quite high numbers of anaerobic nitrogen fixers in the rhizosphere (e.g. 10^6 sulphate reducing nitrogen fixers per gram wet soil (Zuberer & Silver, in press)). On the other hand, Marsho et al (1975) and McRoy et al (1973) deduced that in the aquatic systems they were studying, rhizosphere nitrogen fixation was unlikely to be a significant factor in the growth of macrophytes. This may be because the importance of rhizosphere nitrogen fixation varies between different aquatic ecosystems.

The route for the transfer of nutrients from the rhizosphere to the macrophyte is much more direct than that from root free sediment to either macrophyte or phytoplankton. Rhizosphere nitrogen fixation might therefore contribute a much larger proportion of the nitrogen supply of the primary producers in the lake than does nitrogen fixation in root free sediment. However, if nitrogen fixation does occur in the rhizosphere of aquatic macrophytes, its relative importance to that fixed in other

parts of the ecosystem would obviously depend in part on the relative importance of the macrophytes themselves.

The ecology of the macrophytes in Loch Leven has been changing over recent years. For example, the total biomass of macrophytes has declined, and some species have disappeared whereas others have increased in quantity. This is presumed to be due to the recent rapid eutrophication of the loch (Morgan, 1970; Jupp et al, 1974; Britton, 1974). The only submerged macrophyte which has increased to any great extent is Potamogeton filiformis (Jupp et al, 1974) which has therefore become an important food source for certain species of wildfowl (Allison & Newton, 1974). P. filiformis often grows well under eutrophic conditions, and it must therefore possess some characteristic which makes these conditions suitable for it. Seddon (1972) has shown a relationship between the occurrence of different species of aquatic macrophyte and the trophic levels of a range of lakes (the conductivity of the water was used to represent trophic level), and it seems likely that a wide range of factors combine to determine the optimum trophic level of different plants. Nitrogen fixation in the rhizosphere could be one of these factors, and might thus have contributed to the increase of P. filiformis in Loch Leven.

Gerloff & Krumbholz (1966) suggest that the amount of nitrogen or phosphorus which an aquatic macrophyte growing in a lake contains could indicate whether or not the supply of these nutrients is limiting the growth of the plant. The proportion of the plant's nitrogen which would be supplied by rhizosphere nitrogen fixation would depend on whether nitrogen compounds from other sources were adequate for its growth. However, the plant's overall nutritional status would also affect any nitrogen fixers in the rhizosphere in that it affects the quality and quantity of root exudates, on which the rhizosphere microflora depends for its nutrition. Other physiological factors such as moisture and sunlight also affect the root exudates and hence the microflora. In addition to nitrogen fixation, the microflora can affect the plant in other ways, for example, by phosphate solubilization. (Katznelson, 1965; Nicholas, 1965; Rovira, 1965). Thus a study of the proportion of a plant's nitrogen supplied by nitrogen fixation would be very complex. The experiments described here are but a preliminary attempt to investigate nitrogen fixation in the rhizosphere of P. filiformis.

P. filiformis over-winters by means of tubers buried in the sediment,

PLATE 4-1

Young Potamogeton filiformis plants at Loch Leven : red roots and black sediment.

from which small roots, horizontal rhizomes, and shoots of narrow ribbon-like leaves develop in the spring. The rhizomes often grow along the surface of the sediment. In the parts of Loch Leven where P. filiformis grows, the sediment consists of dense clay overlain by a layer of sand up to 10 cm thick. The surface few millimetres of sediment are reddish in colour, but below this the sediment is grey-black. Plate 4-1 shows some young P. filiformis plants of which the roots can be seen to be red in contrast to the black sediment. In Experiment 1, rates of acetylene reduction by various plant and sediment samples from Loch Leven were compared under a range of conditions. P. pectinatus, samples of which were also included in Experiment 1, is anatomically very similar to P. filiformis, but not so widespread in the loch. In Experiments 2 to 5, it was attempted to verify the results obtained in Experiment 1.

4-2 General Methods

Plants and sediment were dug up between 12 noon and 3.00 p.m. on the day specified from arbitrarily selected sites within the sampling area, and the sediment gently washed off the roots with lake water. In the laboratory in Edinburgh the roots and leaves were separated and the roots were washed three times in distilled water. Samples were placed in $\frac{1}{2}$ or 1 oz bottles which were then sealed as described previously (sections 2-2 and 3-2 respectively). The gas phase was replaced with oxygen free nitrogen (OfN_2) in a variety of ways described in section 4-3. Some methods of gas replacement are likely to be more efficient than others (e.g. those used in Experiments 2, 3 and 5 are likely to be more efficient than those used in Experiments 1 and 4). However, no experiments were carried out to show that this actually was the case. After replacing the gas phase, various amounts of oxygen were added by syringe as required. The oxygen in the bottles was not measured during the experiments, and the amounts quoted in the figures are calculated from the amounts which were added initially. Each treatment contained 3-10 replicates. Ten per cent acetylene was added either immediately or after the vials had been pre-incubated under the assay conditions. The amount of ethylene in the gas phase was determined once as described in section 3-2 after the specified number of hours incubation. The samples

were dried in a 100°C oven, allowed to cool to room temperature, and weighed. Controls were included to determine non-nitrogenase-mediated ethylene production and the ethylene contaminant in the acetylene, which were subtracted from the total ethylene in each bottle.

4-3 Methods particular to individual experiments.

Experiment 1. Samples of P. filiformis and P. pectinatus were collected from Loch Leven on 3rd October 1973. P. filiformis occurs over an area to the north-east of St. Serf's Island and P. pectinatus in a small patch off the west shore of the loch (see Jupp et al, 1974). In the laboratory, leaves, washed leaves, washed roots, clay, and sand were placed in 1 oz bottles. These were sealed and the gas phase was replaced by evacuating and refilling with OfN_2 three times. The bottles were pre-incubated overnight without acetylene under light, dark, anaerobic and semi-aerobic (0.05 atm O_2) conditions at two temperatures (see Table 4-1). The acetylene was then added and the bottles incubated under the same conditions. The ethylene in the gas phase was analysed after 6-10 hours.

Experiment 2. P. filiformis roots were collected from north-east of St. Serf's Island on 15th August 1975 and stored until 27th August 1975 at 4°C. Samples of roots were placed in $\frac{1}{2}$ oz bottles, and the gas phase was replaced by flushing with OfN_2 through a manifold fitted with syringe needles for ten minutes. Acetylene reduction assays were carried out at 0.000, 0.017 and 0.047 atm O_2 and 15°C (lake temperature). The samples were pre-incubated for 24 hours; acetylene was then added and ethylene analysed after 15 further hours incubation under the same conditions.

Experiment 3. P. filiformis roots were collected from north-east of St. Serf's Island on 10th October 1975. The unsealed bottles containing the roots were filled with OfN_2 over water, and then sealed under the water. Acetylene reduction assays were carried out at 0.000, 0.017 and 0.035 atm O_2 and 15°C, after 48 hours incubation with acetylene (no pre-incubation). These samples were used for the isolations described in Sylvester-Bradley (preprint enclosed).

TABLE 4-1

4-1-1a) (EXPERIMENT 1) Potamogeton pectinatus

Sample / nmoles C_2H_4 produced/ g dry weight /h	decaying leaves	green leaves	roots	sand
individual values	0.1118 0.0854 0.1251 0.0397	1.0347 1.7210 0.5822 0.3972	2.0613 23.8498 30.4175 19.3437	0.0012 0.0019 0.0013 0.0029
average	0.0927	0.9338	18.9293	0.0018

4-1-1b) (EXPERIMENT 1) Potamogeton filiformis
Nanomoles C_2H_4 produced / g dry sample / h. Individual values and averages.

Treatment / Sample	0.00 atm O_2		0.05 atm O_2	
	15°C	23°C	15°C	23°C
leaves (in dark)	0.1848 4.6395 0.2245 -	0.3929 0.5955 0.7654 0.3559	0.2057 0.2796 0.3424 0.1508	0.1936 0.3545 0.2928 0.3584
	1.7009	0.5274	0.2446	0.2998
leaves (in light)	-	0.5253 0.3045 0.7853 0.3399	-	0.4391 1.2621 0.9436 0.7928
		0.4888		0.8594
clay	0.0000 0.0031 0.0003 0.0004	0.0048 0.0027 0.0013 0.0017	0.0003 0.0023 0.0003 0.0001	0.0035 0.0015 0.0023 0.0021
	0.0009	0.0026	0.0007	0.0023
sand	0.0025 0.0035 0.0000 0.0000	0.0156 0.0156 0.0068 0.0094	0.0056 0.0000 0.0008 0.0000	0.0070 0.0049 0.0039 0.0030
	0.0015	0.0118	0.0016	0.0047
roots and rhigomes	0.3908 0.5446 0.8373 0.1359	0.4574 0.2826 0.0264 0.2277	2.9611 1.0495 6.0461 14.1842	51.3910 20.5246 59.6565 46.3734
	0.4771	0.2486	6.0602	44.4864

4-1-2 (EXPERIMENT 2) P. filiformis roots.

atm O_2 in gas phase / nmoles C_2H_4 produced/ g dry roots/h	0.000	0.017	0.047
individual values	1.70 2.22 0.43	76.02 80.22 95.73	30.67 43.04 65.99
average	1.45	83.99	46.56

4-1-3 (EXPERIMENT 3) P. filiformis roots.

atm O_2 in gas phase / nmoles C_2H_4 produced/ g dry roots/h	0.000	0.180	0.035
individual values	1.20 0.60 8.29	12.11 24.86 38.25	107.10 90.53 25.50
average	3.36	25.07	74.38

4-1-4 (EXPERIMENT 4) P. filiformis roots.

atm O_2 in gas phase / nmoles C_2H_4 produced/ g dry roots/h	0.00	0.05	0.20
individual values	42.881 56.443 12.859 78.204 30.017 31.963 74.460 33.353 39.789 21.983	4.906 7.252 2.130 18.464 8.633 16.911 - 0.000 1.483 23.349	all negative
average	42.195	9.236	0.000

4-1-5 (EXPERIMENT 5) P. filiformis roots from 5 different sites. Nanomoles C_2H_2 produced / g dry roots / h. Individual values and averages.

site / atm O_2 in gas phase	I	II	III	IV	V
0.00	5.88 13.46 8.36	2.45 15.10 1.63	0.20 1.02 15.71	2.45 2.65 12.24	6.53 1.43 3.67
	9.23	6.39	5.64	5.78	3.88
0.05	7.75 0.41 4.90	10.61 22.64 9.72	8.77 6.53 13.67	8.36 14.69 5.71	0.20 4.49 0.20
	4.35	14.32	9.66	9.59	1.63

OTHER CONDITIONS IN EXPERIMENTS 1 TO 5 (TABLES 4-1-1 TO 4-1-5).

Condition \ Table	4-1-1	4-1-2	4-1-3	4-1-4	4-1-5
Sample collection date	3/10/1973	15/8/1975	10/10/1975	7/8/1974	15/8/1975
Preincubation	overnight	24h(after storage)	none	18h	none
Incubation with C_2H_2 (h)	6-10	15	48	9	15
Atm C_2H_2 in gas phase	0.1				
Atm O_2 in gas phase	see individual tables				
Loch temperature	15°C				
Incubation temperature	15°C except in Experiment 1 (see Table 4-1-1)				
Flushing gas	Of N_2				

Table 4-1 (Experiments 1-5)

Ethylene produced in acetylene reduction assays of freshly samples roots and leaves of Potamogeton filiformis and P. pectinatus, and root-free sediment from Loch Leven, under a range of oxygen tensions from anaerobic (0.00 atm) to aerobic (0.20 atm). Experimental conditions shown in table.

Experiment 4. P. filiformis roots were collected from north-east of St. Serf's Island on 7th August 1974. In the laboratory the roots were placed in 1 oz bottles, and the gas phase was replaced as in Experiment 1. The bottles were pre-incubated for 18 hours at 0.00, 0.05 and 0.20 atm O_2 and 15°C. Acetylene was then added and the bottles were incubated for nine further hours under the same conditions after which the gas phase was analysed for ethylene.

Experiment 5. On 15th August 1975 P. filiformis roots were collected from five different sites north-east of St. Serf's Island. The gas phase in the bottles containing the samples was replaced as in Experiment 2, and acetylene reduction assays carried out at 0.00 and 0.05 atm O_2 without pre-incubating. Ethylene in the gas phase was analysed after 15 hours.

4-4 Results, discussion and suggestions for further work.

The results are shown in Table 4-1. It can be seen in Table 4-1-1 that the roots and rhizomes of both P. pectinatus and P. filiformis produced much more ethylene than the other samples, and the effect of oxygen levels on this activity was considerable (see P. filiformis "roots and rhizomes"). Temperature also affected ethylene production by the roots. The Potamogeton roots in all the experiments were red in colour in contrast to the grey-black of the clay and sand they were embedded in (Plate 4-1). The plant also possesses an aerenchyma, and as explained elsewhere (Sylvester-Bradley, preprint enclosed) the rhizosphere is therefore presumably aerated. Thus it is a potential habitat for aerobic microbial activity, even though the root free sediment is anaerobic. There was also some light-dependent activity on the leaves, but this was much lower than the activity on the roots, and may have been caused by the heterocystous blue-green algae which were abundant in the loch at the time of sampling. In Table 4-1-2 it can be seen that the optimum oxygen concentration for ethylene production by roots and rhizomes was above 0.000 and below 0.047 atm, which is much lower than the proportion of oxygen in air (0.2 atm), and the root samples in Table 4-1-3 also produced more ethylene under semi-aerobic than

anaerobic conditions.

The results in Table 4-1-1, 4-1-2 and 4-1-3 are therefore consistent with the theory that microaerophilic organisms are responsible for nitrogenase activity on the roots. However, in Table 4-1-4 it can be seen that ethylene production was higher in the anaerobic treatment, and this was also the case in other experiments carried out in 1974 (not shown). The results in Table 4-1-5 are extremely variable and the experimental treatments had no effect. In yet other experiments (not shown) no ethylene was produced at all. None of the different flushing methods seemed to significantly alter this variability, although in some cases pre-incubation seemed to reduce it. For example, the samples in Experiments 2 and 5 were collected on the same day and yet the results are completely different.

There are various possible explanations for these inconsistencies in the results. Firstly, the roots of terrestrial plants on which asymbiotic nitrogen fixers are active are very sensitive to exposure to air when sampling (Dobereiner et al, 1972a & b; Harris & Dart, 1973). These authors observed a lag before the onset of acetylene reduction which could be shortened if the roots were removed from the soil under anaerobic conditions in a glove box, or if nitrogen fixation was measured in intact plant-soil systems. It is possible that while sampling the Potamogeton roots similar damage occurred. The time taken to recover from this damage would vary depending on how serious it was and the physiological state of the plants. Secondly, the oxygen sensitivity of aerobic nitrogen fixers may depend on the conditions under which they are grown. For example, the oxygen sensitivity of Azotobacter chroococcum varies depending on its nutrient supply (Dalton & Postgate, 1968 & 1969) and the oxygen sensitivity of acetylene reducing spirilla isolated from Potamogeton roots was shown to decrease in the presence of amino acids (enclosed preprint). Thus the oxygen sensitivity of aerobic nitrogen fixers in the rhizosphere may depend on the quality and quantity of the root exudates. Thirdly, although the conditions were made as anaerobic as possible some oxygen may have remained, for example in the root tissues. The higher rates of ethylene production under "anaerobic conditions" in Table 4-1-4 could therefore have been due to microaerophilic organisms.

Fourthly, nitrogen fixation associated with plants is likely to undergo diurnal and seasonal fluctuations, which might have caused some of the variability. Thus it can be seen that despite the variability of the results of the acetylene reduction assays, microaerophilic spirilla could be responsible for the nitrogenase activity of the roots. However, it is also possible that the variability represents a change in the type or number of nitrogen fixing organisms on the roots. It is therefore important that a more detailed microbiological investigation of the nitrogen fixing organisms in the rhizosphere should be carried out. To actually prove that spirilla were responsible for nitrogen fixation on the roots it would be necessary to obtain a sterile Potamogeton plant and inoculate it with a Spirillum culture. Attempts to grow sterile Potamogeton plants from surface sterilized tubers failed: all the plants which grew reduced acetylene without having been inoculated. Seeds would be less likely to have an internal microflora than tubers. An attempt to grow sterile plants from seeds might therefore be profitable.

It is clearly not possible to make any predictions as to the quantitative significance of rhizosphere nitrogen fixation from these results. Apart from their inconsistency, comparisons between results from different samples on a dry weight basis do not give a realistic estimate of their relative significance, because different samples vary in their relative abundance and water content. Furthermore, it appears that to uproot the plants in order to measure their nitrogenase activity is more vigorous treatment than they are able to tolerate. When used on sediment, the acetylene reduction assay suffers because of the slow diffusion of gases in and out of the sample, and it seems that $^{15}N_2$ would diffuse equally slowly, if not more so (section 2-5). However, the measurement of ^{15}N-enrichment from $^{15}N_2$ does not depend on the diffusion of a gaseous product out of the sample. Also, the aerenchyma is an especially adapted system for the transport of gases to the roots. Yoshida & Broadbent (1975) showed that $^{15}N_2$ in the atmosphere was transported to the rhizosphere of rice plants. Therefore it seems that measurement of ^{15}N-enrichment of plants and rhizosphere sediment which had been incubated under water containing $^{15}N_2$ might give a much more realistic estimate of the quantity of nitrogen being fixed than the acetylene reduction assay. There would still be problems in estimating the amount being fixed in root free sediment, and it seems that

measurements of rhizosphere nitrogen fixation would be best considered separately. Comparisons could be made between different plants growing under different conditions, and also between lakes which were colonized by macrophytes to differing extents. Thus a picture could be built up of the relative significance of rhizosphere nitrogen fixation in different lakes.

4-5 Summary of main conclusions.

It was shown that ethylene was produced in acetylene reduction assays of roots of Potamogeton filiformis and P. pectinatus at much higher rates than the leaves or root free sediment (up to 100 nmoles ethylene/g dry weight/h at lake temperature). A microaerophilic acetylene reducing spirillum was isolated from roots of P. filiformis which produced more ethylene under semi-aerobic than anaerobic conditions (enclosed preprint). This organism may be responsible for supplying significant amounts of nitrogen to P. filiformis. However, the oxygen concentration at which maximum rates of ethylene production in acetylene reduction assays of the roots occurred varied within and between some of the experiments. This might be due to variations in the oxygen sensitivity and activity of spirilla on the roots, but it is also possible that the population of nitrogen fixing organisms on the roots changes, and more detailed microbiological investigations are needed to determine whether this is the case. It seems that ^{15}N-enrichment of plants from $^{15}N_2$ supplied over intact plant-sediment systems would be a more accurate method of assessing the quantitative significance of nitrogen fixation in the rhizosphere of P. filiformis than the acetylene reduction assay.

CHAPTER 5. GENERAL DISCUSSION

5-1 Implications of the results for the quantitative and qualitative measurement of nitrogen fixation in lake sediments.

The acetylene reduction assay was tested to determine whether or not it could be used to measure nitrogen fixation in lake sediments quantitatively (Chapter 2). Three different factors which interfere directly with quantification of acetylene reduction rates were discovered: 1) Ethylene absorption by the sediment is inhibited in the presence of acetylene (Sylvester-Bradley et al, 1974). It is therefore not possible to measure non-nitrogenase-mediated ethylene production, which may occur in any system containing an unknown mixture of organisms. This would cause over-estimation of the rate of nitrogen fixation (rather than underestimation as suggested by Flett et al, 1975). 2) Nitrogen fixing methane oxidizers which do not reduce acetylene may be active in the sediment. This would cause under-estimation of the rate of nitrogen fixation. 3) The rate of acetylene diffusion into unshaken sediment samples appears to limit the rate of acetylene reduction. Inhibition of acetylene reduction by nitrogen gas was also observed, but this might be overcome if the nitrogenase was saturated with acetylene. Both these factors would cause under-estimation of nitrogen fixation.

It seems likely that the overall effect of these factors would be under-estimation of the rate of nitrogen fixation in lake sediments as measured by the acetylene reduction assay, unless the rate of non-nitrogenase-mediated ethylene production is very high. However, even if the acetylene reduction assay was a quantitative measure of nitrogen fixation in a given system, it would not be possible to extrapolate from the results to the ecosystem from which the samples had been taken unless all the variations in environmental parameters and the population of nitrogen fixers could be taken into account. Qualitative information regarding these variables is therefore required. In Chapter 3, ethylene production rates in acetylene reduction assays of sediments collected from three eutrophic British lakes (Loch Leven, Esthwaite Water and Grasmere) at different times of year were compared. It was found that sediment from Loch Leven produced more ethylene if it was black than if it was brown, and contrary to the results of Howard et al (1970), at higher temperatures. From experiments carried out to compare the numbers of endospore forming nitrogen fixers present in black and brown

Loch Leven sediment, it seemed that they might be responsible for the ethylene production observed. However, other nitrogen fixers were also present, and further investigations into their relative significance are required. The highest rates of ethylene production of all were observed in Esthwaite and Grasmere sediments collected two weeks after the overturn, and it seemed that this might be due to a different group of organisms than the ethylene produced from Loch Leven sediments. Thus it can be seen that there may be fundamental differences between nitrogen fixation in sediments of stratifying and non-stratifying lakes. In view of these variations in ethylene production both within and between sediments of different lakes, it is clear that even if using a quantitative method of measurement, more work is needed on the qualitative aspects of nitrogen fixation in lake sediments before quantitation of nitrogen fixation in the whole ecosystem could be carried out. This conclusion casts doubt on the accuracy of the quantitative estimates in previously published reports of investigations into rates of nitrogen fixation in lake sediments, particularly in cases where the acetylene reduction assay was used. The qualitative results are still of interest, however.

Previously published reports that acetylene reduction rates (e.g. Brooks et al, 1971) and also microbial activity in general (e.g. Henrici & McCoy, 1938) decrease with depth in the sediment should also be considered in view of the way in which the results are expressed; in Chapter 3 the change with depth of the observed rates of ethylene production by Esthwaite and Grasmere sediments was quite different when the results were expressed per gram dry weight than when they were expressed per gram wet weight. This is simply a consequence of the fact that % dry weight of sediment increases with depth. When discussing a change of activity with depth in sediments, probably the least ambiguous way to express the results is per m^2 sediment (e.g. Whitney et al, 1975).

Future work on nitrogen fixation in root free sediments should be aimed a) to improve the acetylene reduction assay for measurement of nitrogen fixation and b) to investigate further the quality of nitrogen fixation, for example by comparison of rates with direct measurements of parameters such as Eh, carbon and nitrogen levels, and more detailed microbiological investigations, including nitrogen fixing methane oxidizers. This approach should in the long run produce more meaningful results than

non-critical attempts to quantitate nitrogen fixation, even though it may initially appear to be less appropriate.

In view of the observed variations in ethylene production rates between different sediments and in response to temperature and oxygen levels, and the fact that it was stimulated by glucose and inhibited by trichloroacetic acid, it has been assumed that it is a biologically mediated reaction. Although the possibility of abiological acetylene reduction is difficult to eliminate entirely, because most methods of sterilization have chemical effects and may not be immediately active, the assumption is considered reasonable for the purposes of this thesis.

In Chapter 4 it is shown that ethylene was produced in acetylene reduction assays of roots of Potamogeton filiformis and P. pectinatus from Loch Leven at much higher rates than by the leaves or root free sediment, and in some experiments on P. filiformis roots these rates were higher at low oxygen tensions than either fully aerobic or anaerobic conditions. Microaerophilic spirilla which reduce acetylene and grow in nitrogen free media were isolated from these roots, and such organisms may provide a significant proportion of the nitrogen supply of P. filiformis in the loch (Sylvester-Bradley, enclosed preprint). In the summer the nitrate levels in the loch water are very low (less than 0.1 ug/ml) and nitrogen fixation in the rhizosphere of P. filiformis may be a contributary factor to the recent increase in biomass of this plant, which occurred despite an overall decline of macrophytes in the loch. It is concluded that nitrogen fixation by spirilla in the rhizosphere of aquatic macrophytes may be more generally significant than has so far been shown, and further investigations are needed into the distribution both of nitrogen fixing spirilla and other nitrogen fixers in the rhizosphere of a wider range of plants. The problems encountered in obtaining consistent results in acetylene reduction assays of the roots might be due to the sensitivity of the roots to exposure to air, and it might be possible to overcome this by using ^{15}N-enrichment of plants from $^{15}N_2$ in intact plant-sediment systems as an alternative method to acetylene reduction assays. When nitrogen fixation is being studied in lake sediments as a whole, the relative importance of rhizosphere and root free sediment should be taken into account.

5-2 Speculations.

By making many assumptions, it is possible to calculate the theoretical amount of ethylene which would be produced in acetylene reduction assays of a system which was fixing a given amount of nitrogen. This figure can then be compared with the actual results obtained. This comparison is useful in that it can be used to show whether, considering the limitations of the acetylene reduction assay, the rates of ethylene production measured in the system under investigation are at all likely to be significant in the ecosystem.

For example, the average nitrogen content of Potamogeton filiformis from near St. Serf's Island is 16.9 mg N/g dry weight (total Kjeldahl nitrogen; the values decrease from May to August by 50%; Dr. E. Dale Allen pers. comm.). Therefore, assuming that no nitrogen fixation occurs on the leaves, and the roots constitute 10% of the total plant tissue, 84.5 mg N/g dry roots would need to be fixed and transferred to the plants to supply 50% of their nitrogen (assuming no nitrogen turnover occurs during the year). Assuming the 3:1 molar conversion ratio between ethylene produced and molecular nitrogen fixed ($3\ C_2H_2 + 6\ H^+ + 6\ e^- \rightarrow 3\ C_2H_4$; $N_2 + 6\ H^+ + 6\ e^- \rightarrow 2\ NH_3$) this would be the equivalent of 400 nanomoles ethylene/g dry roots, for the six months of the growing season. However, if the wildfowl eat 50% of the Potamogeton as suggested by Jupp & Spence (1974) this amount must be increased twofold. Thus if rhizosphere nitrogen fixation were to supply all the plants' nitrogen, in theory 800 nanomoles ethylene/g dry roots/h would have to be produced. The highest individual rate of ethylene production observed by roots was 154 nmoles ethylene/g dry roots/h. Thus unless the ethylene production results under-estimate nitrogen fixation considerably, it seems that it can only be supplying a proportion of the plant's nitrogen is fixed, which may or may not be transferred to the plant. For root free sediment, 100 Kg N fixed/ha/year in a 15 cm thick layer of sediment would theoretically be equivalent to 0.8143 nmoles ethylene/ml sediment/h. (Invertebrates were observed to at least 15 cm depth in sediment cores). If the sediment contains 20% dry weight, about 4 nmoles ethylene/g dry weight/h would have to be produced. This is similar to the amounts detected in the experiments described in this thesis, and since they are in fact likely to be an

under-estimate, it is possible that 100 Kg N could be fixed/ha/year.

Clearly the significance of these amounts of nitrogen to the nitrogen balance of the lake depends on the area of the sediment, the amount of nitrogen flowing into the lake from external sources, and the extent to which the nitrogen fixed is transferred to other organisms. The average weekly nitrogen input from streams into Loch Leven was 5802 Kg between April 1971 and March 1972 (Holden & Caines, 1974). There are no such data for Esthwaite Water. The respective surface areas of Loch Leven and Esthwaite Water are 1331 and 100 ha. Grasmere is rather different because it has recently started receiving the effluent from Grasmere village sewage works and the lake is consequently changing rapidly; the significance of nitrogen fixation is thus also likely to be changing. In Loch Leven, 100 Kg N/ha/year is 44% of the total nitrogen input in streams in 1971-1972. Thus, if transfer to other organisms occurs, it appears that nitrogen fixation in the sediment could be a significant source of nitrogen for biological productivity in the loch.

On the basis of the qualitative results described in section 5-1 it is also possible to speculate on the different types of lake in which nitrogen fixation in the sediments is most likely to be significant. For example, in small ponds colonized by dense stands of rooted plants, more rhizosphere than algal nitrogen fixation would be expected. However, in Loch Leven, where it seems that algae are competing successfully with macrophytes, algal nitrogen fixation might be greater: blooms of heterocystous blue-green algae have recently occurred in Loch Leven (Bailey-Watts, 1974). The importance of root free nitrogen fixation would be expected to depend on whether or not the lake undergoes temperature stratification, and the Eh of the sediment. It is possible that more nitrogen fixation occurs in eutrophic than oligotrophic lakes, as the Eh of the sediments of eutrophic lakes may be lower as a consequence of the availability of more respirable organic matter. If this were the case, it would mean that nitrogen fixation in lakes constituted a positive feed back system, and thus that nitrogen fixation itself is not necessarily an adaptive advantage to nitrogen fixing organisms in lakes.

5-3 Application.

In view of the suggestion that the study of nitrogen cycle in

lakes is of use to man because of the problem of cultural eutrophication (e.g. Keeney, 1973), and the numerous attempts to quantify nitrogen fixation in lakes in order to construct nitrogen budgets, it seems important to point out again that this should not be the only aim of such investigations. Assuming that the aim of an applied scientist is to produce useful results, it seems that in order to construct a realistic and therefore more useful nitrogen budget, it is better to obtain both quantitative and qualitative data, and investigations into nitrogen fixation in lake sediments should include observations as to the qualitative nature of the sediment and the microorganisms in it.

Furthermore, it seems that the applicability of studies of the nitrogen cycle to the problem of eutrophication may not be as immediate as it appears to be. Cultural eutrophication has already been overcome in some lakes by the measures suggested by Vollenweider (1968), by dredging out the sediment which would otherwise continue releasing nutrients for a long time after the external sources had been eliminated, or by the aeration of the anaerobic hypolimnion (e.g. Bengtsson & Gelin, 1975). Thus it seems reasonable to suggest that in its present context the study of nitrogen fixation in lake sediments is a pure rather than an applied science.

5-4 Microbial ecology - remarks.

The difference between the study the ecology of microbes and that of other living organisms is that microbes are invisible to the naked eye, whereas other organisms are not. Microbiological methods therefore involve more manipulation of the system, and hence disturb it more. Different methods will disturb the system in different ways, so the combination of several different methods for studying the same problem seems the best way of overcoming this difficulty.

Secondly, the system being studied is very complex and it is therefore difficult to design an experiment which will have conclusive results. To quote Collins (1963) "Looking at a glass beaker full of lake water and knowing what to do with it are two completely different things, . . .". In fact, it is found that it is necessary to carry out several inconclusive experiments before any conclusive results are obtained.

These problems make microbial ecology slow and laborious. However, the few conclusions which can be drawn are rewarding because they could never be replaced by experiments on the simpler systems of which the whole ecosystem is made up.

FIG I-1

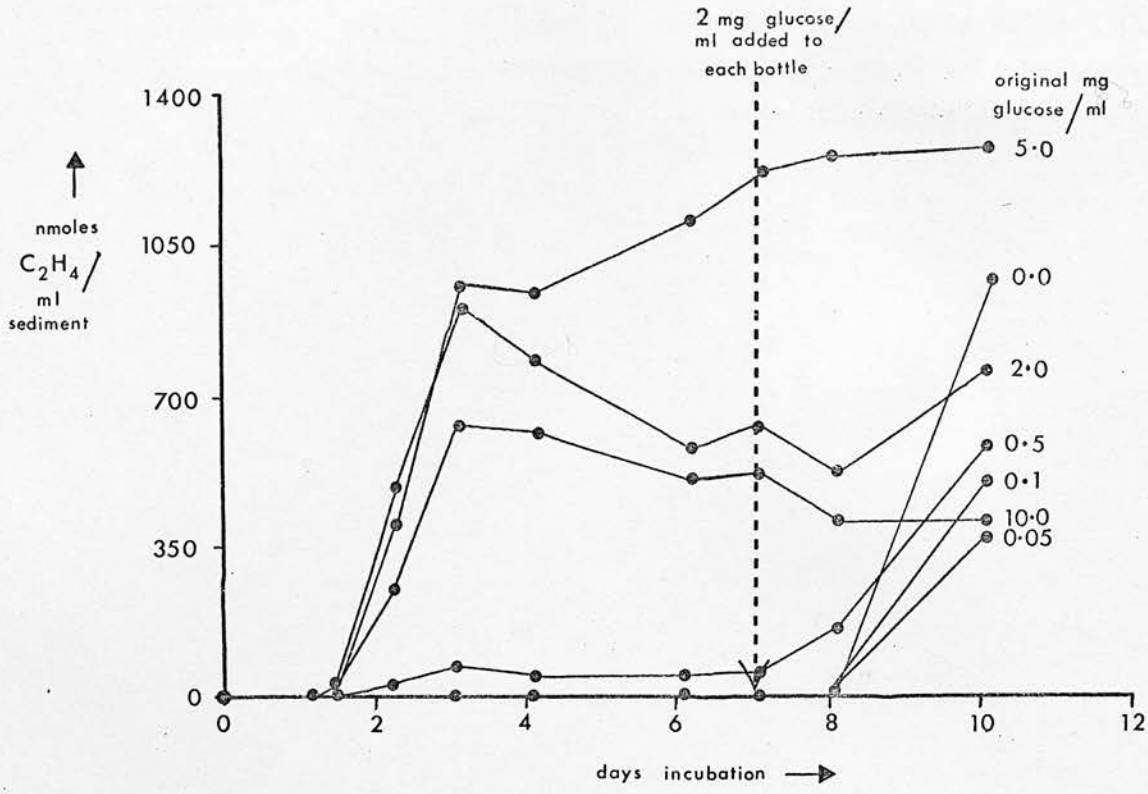

2 mg glucose/ml added to each bottle
original mg glucose/ml
5·0
0·0
2·0
0·5
0·1
10·0
0·05
1400
1050
700
350
0
0
2
4
6
8
10
12
nmoles C2H4/ml sediment
days incubation

FIG I-2

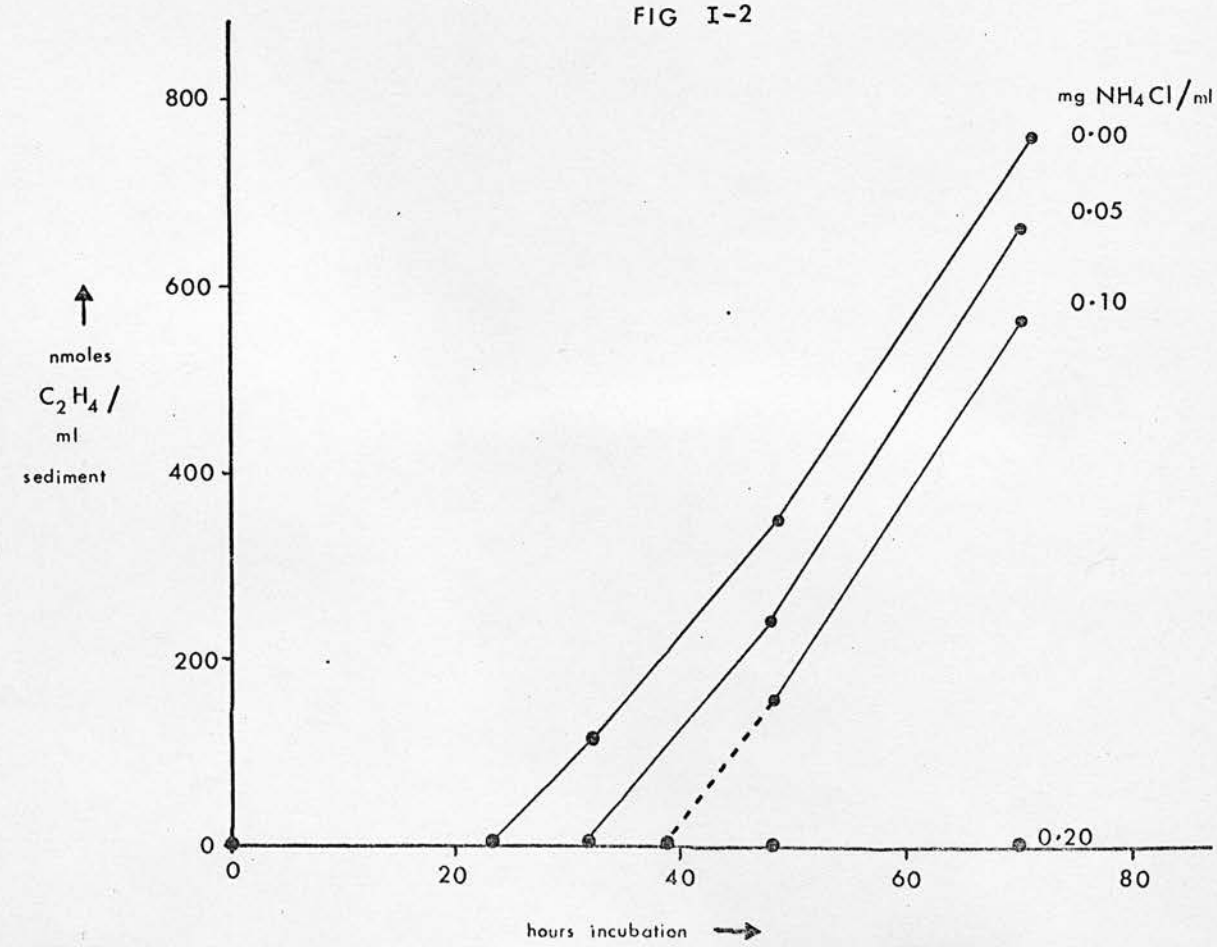

mg NH4Cl/ml
0·00
0·05
0·10
0·20
800
600
400
200
0
0
20
40
60
80
nmoles C2H4/ml sediment
hours incubation

Figure I-1

Ethylene produced in acetylene reduction assays of Loch Leven Reed Bower sediment ammended with various concentrations of glucose after 0 and 7 days incubation at 25°C. Points are means of three replicates.

Figure I-2

Ethylene produced in acetylene reduction assays of Loch Leven Reed Bower sediment ammended with 5 mg glucose/ml and various concentrations of NH_4Cl, and incubated at 25°C. Points are means of three replicates.

APPENDIX I. EXPERIMENTS ON ACETYLENE REDUCTION BY GLUCOSE AMMENDED SEDIMENTS.

In preliminary experiments on sediments collected through about 3 m depth of water south-west of Reed Bower (see map; Figure 3-1) it was found that no acetylene reduction occurred. A series of experiments on glucose ammended sediment, which did reduce acetylene, was therefore carried out. Two of these experiments are reported here.

Materials and Methods. Approximately 3 ml samples of sediment were placed in wide-necked 1 oz bottles in triplicate for each treatment. The bottles were sealed and flushed out with oxygen free nitrogen. A total of 2 ml of glucose solution, and/or NH_4Cl solution and/or water were added to the sediment to give the concentrations shown in the graphs. Twenty per cent acetylene was added and the bottles were incubated at 25°C. Gas samples (0.2 ml) were withdrawn at time intervals and analysed on a Pye 104 gas chromatograph as described in section 3-2. In Experiment 1 0.2 mg glucose/ml sediment were added to all the bottles after seven days incubation.

Results and Discussion. The results are shown in Figures I-1 and I-2. In Figure I-1 it can be seen that glucose addition stimulated ethylene production after a lag of $1\frac{1}{2}$ days. The optimum glucose concentration for ethylene production was 5 mg/ml sediment. When 0.2 mg/ml glucose were added after seven days incubation, ethylene production was stimulated in all the bottles after a lag of one day. This implies that the stimulation was a response to glucose rather than any other factor.

Figure I-2 shows the effect of adding NH_4Cl to the sediment as well as glucose. With 0.2 mg NH_4Cl/ml no ethylene at all was produced within three days. The addition of 0.1 mg and 0.05 mg NH_4Cl/ml lengthened the lag phase but after this period the rate of ethylene production was as high as with no added NH_4Cl. This implies that because NH_4Cl inhibits nitrogen fixation, the onset of nitrogen fixation is delayed until glucose-stimulated biological activity in the sediment has removed the added NH_4Cl. The lag phase which occurs even in the absence of added NH_4Cl might reflect a similar effect on the nitrogen compounds naturally present in the sediment.

Conclusion. The results imply that nitrogen fixation in the lake sediment samples tested is limited by the availability of energy substrates and by high natural levels of fixed nitrogen in the sediment. This may reflect

the need of nitrogen fixation for a high environmental C:N ratio (Huser, 1965). However, the conditions in these experiments were artificial in that the ethylene production may have been caused by bacteria lying dormant in the sediment until the glucose was added. Further experiments were therefore carried out on unammended sediments known to contain different natural levels of organic carbon (Chapter 3). Ammendment of sediments with energy substrates would be a useful approach to adopt once natural rates of nitrogen fixation had been determined. Substrates specific to particular species of nitrogen fixer could be added and their effect on the natural rate of ethylene production determined. If one substrate increased the rate of ethylene production more immediately than the others, this would indicate which species of nitrogen fixer was responsible for the ethylene production.

APPENDIX II. COMPARISON OF RATE OF ETHYLENE PRODUCTION IN ACETYLENE REDUCTION ASSAYS AND RATE OF ^{15}N-ENRICHMENT FROM $^{15}N_2$ BY LAKE SEDIMENT.

This experiment was designed to determine the ratio of C_2H_2 reduced to N_2 fixed by lake sediment. However, from previous experiments (Chapter 2) it appeared that the rate of C_2H_2 reduction was limited by the rate of C_2H_2 diffusion into the sediment. Thus it seemed likely that the rate of ^{15}N-enrichment would be similarly affected.

Materials and Methods. Brown and black sediment were collected from Loch Leven North Deep with a Jenkin's Corer. On the same day acetylene reduction assays were carried out on triplicate samples of each sediment type under anaerobic conditions at 15°C in the dark as described previously (section 2-2).

The ^{15}N-enrichment experiment was also set up on the same day as follows: Four sets of triplicate 2 ml samples of black and brown sediment were placed in bottles under anaerobic conditions using He as the flushing gas using the same methods and materials as for the acetylene reduction assays. One set of bottles was then evacuated to 0.5 atm (measured on a Hg manometer). Seven ml 99 atom% excess $^{15}N_2$ from a glass sphere (obtained from Prochem-BOC, Deer Park Road, London SW19 3UF) were then added with a He-sparged syringe (this gave approximately 0.5 atm each of He and $^{15}N_2$ in the gas phase). Enough He was added with a syringe to remove any negative pressure inside the bottles. The gas phase was mixed by pumping the syringe, and a 2 ml gas sample removed and injected into a vial over He-flushed water, which was then sealed. Seven ml He-flushed water were added to the glass sphere to restore the original gas pressure.

All the bottles were then incubated at 15°C in the dark, and two further sets of bottles exposed to $^{15}N_2$ as above after 18 hours and 39 hours. The fourth set of bottles was not exposed to $^{15}N_2$ (control). After 48 hours a further 2 ml gas sample was removed from each bottle and stored as above, and the bottles placed in a deep freeze to stop the reaction.

The analyses were carried out as follows: After thawing sediment samples were transferred to 50 ml Kjeldahl flasks with 3 ml conc. H_2SO_4, distilled water, and one Kjeltab. (Thompson & Capper, Liverpool) containing 2.35 g K_2SO_4, 1.25 g $CuSO_4$ and 25 mg Se, and digested for 25 minutes after clearing. When cool 10 ml 40% (10N) NaOH were added to each sample which was then steam distilled into 10 ml 0.1 N HCl for exactly 3 minutes after the colour changed. The samples were made up to 50 ml with distilled water, 2 ml were removed for estimation of total nitrogen, and the remainder evaporated down to 1 ml on a sand bath.

Mass spectrometric analyses on the distillates and gas samples were carried out by Dr. Tony Haystead (H.F.R.O., Bush).

Results and Discussion. The results of the acetylene reduction assays showed that 0.37 nmoles ethylene/h/ml black sediment and 0.21 nmoles ethylene/h/ml brown sediment were produced.

The mass spectrometric analyses showed no enrichment of the sediment, even after $2\frac{1}{2}$ days incubation, and although the mass spectrometer would have detected 0.001 atom% excess of ^{15}N over a natural level of 0.336%. This could have been due to a number of reasons, for example, slow rate of diffusion of $^{15}N_2$ into the sediment as predicted (Rice & Paul (1971) had similar problems with waterlogged soil), or that the ^{15}N fixed was diluted in the sediment by a very high original total N content, or that denitrifying activity was very high. Clearly this experiment should be repeated using shaken diluted sediment for both acetylene reduction and ^{15}N-enrichment assays. This would show whether slow diffusion rates are the cause of the inconclusive results.

References.

Abeles, F.B. (1973) Ethylene in Plant Biology. New York and London: Academic Press.

Abeles, F.B., Craker, L.E., Forrence, L.E. & Leather, G.R. (1971) Fate of air pollutants: Removal of ethylene, SO_2 and NO_2 by soil. Science. 171, 914-916.

Allison, A. & Newton, I. (1974) Waterfowl at Loch Leven, Kinross. Proceedings of the Royal Society of Edinburgh (B). 74, 365-381.

Babiuk, L.A. & Paul, E.A. (1970) The use of fluorescin isothiocyanate in the determination of the bacterial biomass of grassland soil. Canadian Journal of Microbiology. 16, 57-62.

Bailey-Watts, A.E. (1974) The algal plankton of Loch Leven, Kinross. Proceedings of the Royal Society of Edinburgh (B). 74, 135-156.

Barrow, N.J. & Jenkinson, D.S. (1962) The effect of water-logging on fixation of nitrogen by soil incubated with straw. Plant and Soil. 16, 258-262.

Benstsson, L. & Gelin, C. (1975) Artificial aeration and suction dredging methods for controlling water quality. In The effects of storage on water quality. Papers and Proceedings of Symposium published by Water Research Centre, Marlow, Bucks, SL7 2HD.

Bergerson, F.J. (1970) The quantitative relationship between nitrogen fixation and the acetylene reduction assay. Australian Journal of Biological Sciences. 23, 1015-1025.

Bird, C.W. & Lynch, J.M. (1974) Formation of hydrocarbons by micro-organisms. Chemical Society Reviews. 3, 309-328.

de Bont, J.A.M. & Mulder, E.G. (1974) Nitrogen fixation and co-oxidation of ethylene by a methane-utilizing bacterium. Journal of General Microbiology. 83, 113-121.

de Bont, J.A.M. (1975) Oxidation of ethylene by bacteria. Annals of Applied Biology. 81, 119-121.

Bristow, J.M. (1974) Nitrogen fixation in the rhizosphere of freshwater angiosperms. Canadian Journal of Botany. 52, 217-221.

Britton, R.H. (1974) Factors affecting the distribution and productivity of emergent vegetation at Loch Leven, Kinross. Proceedings of the Royal Society of Edinburgh (B). 74, 209-218.

Brooks, R.H., Brezonik, P.L., Putnam, H.D. & Keirn, M.A. (1971) Nitrogen fixation in an estuarine environment: the Waccasassa on the Florida Gulf coast. Limnology and Oceanography. 16, 701-711.

Brouzes, R. & Knowles, R. (1971) Inhibition of growth of Clostridium pasteurianum by acetylene: implication for nitrogen fixation assay. Canadian Journal of Microbiology. 17, 1483-1489.

Brouzes, R. & Knowles, R. (1973) Kinetics of nitrogen fixation in a glucose ammended, anaerobically incubated soil. Soil Biology and Biochemistry. 5, 223-229.

von Bulow, J.F.W. & Dobereiner, J. (1975) Potential for nitrogen fixation in maize genotypes in Brazil. Proceedings of the National Academy of Sciences of the U.S.A. 72, 2389-2396.

Calvert, S.E. (1974) The distribution of bottom sediments in Loch Leven, Kinross. Proceedings of the Royal Society of Edinburgh (B). 74, 69-80.

Campbell, N.E.R. & Evans, H.J. (1969) Use of Pankhurst tubes to assay acetylene reduction by facultative and anaerobic nitrogen-fixing bacteria. Canadian Journal of Microbiology. 15, 1342-1243.

Chen, R.L., Keeney, D.R., Graetz, D.A. & Holding, A.J. (1972) Denitrification and nitrate reduction in lake sediments. Journal of Environmental Quality. 1, 158-162.

Collins, V.G. (1960) The distribution and ecology of gram-negative organisms other than entrobacteriaceae in lakes. Journal of Applied Bacteriology. 23, 510-514.

Collins, V.G. (1963) The distribution and ecology of bacteria in freshwater. Proceedings of the Society of Water Treatment and Examination. 12, 40-73.

Cooke, G.W. & Williams, R.J.B. (1970) Losses of nitrogen and phosphorus from agricultural land. Proceedings of the Society of Water Treatment and Examination. 19, 253-276.

Cox, R.M. & Fay, P. (1969) Special aspects of nitrogen fixation by blue-green algae. Proceedings of the Royal Society of London (B). 172, 357-366.

Dalton, H. & Postgate, J.R. (1968) Effect of oxygen on growth of Azotobacter chroococcum in batch and continuous cultures. Journal of General Microbiology. 54, 463-473.

Dalton, H. & Postgate, J.R. (1969) Growth and physiology of Azotobacter chroococcum in continuous culture. Journal of General Microbiology. 56, 307-319.

Dilworth, M.J. (1966) Acetylene reduction by nitrogen-fixing preparations from Clostridium pasteurianum. Biochimica et Biophysica Acta. 127, 285-294.

Dobereiner, J. (in press) N_2-fixing bacteria in the rhizosphere.

Dobereiner, J., Day, J.M. & Dart, P.J. (1972a) Nitrogenase activity and oxygen sensitivity of the Paspalum notatum - Azotobacter paspali association. Journal of General Microbiology. 71, 103-116.

Dobereiner, J., Day, J.M. & Dart, P.J. (1972b) Nitrogenase activity in the rhizosphere of sugar cane and some other tropical grasses. Plant and Soil. 37, 191-196.

Dobereiner, J., Day, J.M. & Dart, P.J. (1973) Rhizosphere associations between grasses and nitrogen-fixing bacteria: effect of O_2 on nitrogenase activity in the rhizosphere of Paspalum notatum. Soil Biology and Biochemistry. 5, 157-159.

Dugdale, V.A. & Dugdale, R.C. (1962) Nitrogen metabolism in lakes. II Role of nitrogen fixation in Sanctuary Lake Pennsylvania. Limnology and Oceanography. 7, 170-175.

Edmondson, W.T. & Winberg, G.G. (eds.) (1971) A manual on methods for the assessment of secondary productivity in fresh waters. IBP Handbook No. 17. Blackwell Scientific Publications.

Fay, P. (1965) Heterotrophy and nitrogen fixation in Chlorogloae fritschii. Journal of General Microbiology. 39, 11-20.

Fay, P., Stewart, W.D.P., Walsby, A.E. & Fogg, G.E. (1968) Is the heterocyst the site of nitrogen fixation in blue-green algae? Nature London. 220, 810-812.

Flett, R.J., Hamilton, R.D. & Campbell, N.E.R. (in press) Nitrogen fixation in aquatic environments - A critical study of the acetylene reduction assay.

Flett, R.J., Rudd, J.W.M. & Hamilton, R.D. (1975) Acetylene reduction assays for nitrogen fixation in freshwater: a note of caution. Applied Microbiology. 29, 580-583.

Gambarian, M.E. (1958) Assimilation of molecular nitrogen in the water and soil of the Sevan lake. Microbiology. 27, 360-363.

Gerloff, G.C. & Krumbholz, P.M. (1966) Tissue analysis as a measure of nutrient availability for the growth of angiosperm aquatic plants. Limnology and Oceanography. 11, 529-537.

Goering, J.J. & Neess, J.C. (1964) Nitrogen fixation in 2 Wisconsin Lakes. Limnology and Oceanography. 9, 530-539.

Hardy, R.W.F., Burns, R.C. & Holsten, R.D. (1973) Applications of the acetylene-ethylene assay for measurement of nitrogen fixation. Soil Biology and Biochemistry. 5, 47-81.

Hardy, R.W.F., Holsten, R.D., Jackson, E.K. & Burns, R.C. (1968) The acetylene-ethylene assay for N_2 fixation: laboratory and field evaluation. Plant Physiology. 43, 1185-1207.

Harris, D. & Dart, P.J. (1973) Nitrogenase activity in the rhizosphere of Stachys sylvatica and some other dicotyledenous plants. Soil Biology and Biochemistry. 5, 277-279.

Harris, R.F. & Sommers, L.E. (1968) Plate-dilution frequency technique for assay of Microbial Ecology. Applied Microbiology. 16, 330-334.

Hayes, F.R. & Anthony, E.H. (1959) Lake Water and Sediment VI The standing crop of bacteria and its place in the classification of lakes. Limnology and Oceanography. 4, 299-315.

Head, W.D. & Carpenter, E.J. (1975) Nitrogen fixation associated with the marine macroalga.Codium fragile. Limnology and Oceanography. 20, 815-823.

Henrici, A.T. & McCoy, E. (1938) The distribution of heterotrophic bacteria in the bottom deposits of some lakes. Transactions of the Wisconsin Academy of Sciences, Art and Letters. 31, 323-361.

Heron, J. (1961) The seasonal variations of phosphate, silicate, and nitrate in waters of the English Lake District. Limnology and Oceanography. 6, 338-346.

Hill, S. (1975) Acetylene reduction by Klebsiella pneumoniae in air related to colony dimorphism on low fixed nitrogen. Journal of General Microbiology. 91, 207-209.

Hill, S. & Postgate, J.R. (1969) Failure of putative nitrogen-fixing bacteria to fix nitrogen. Journal of General Microbiology. 58, 277-285.

Holden, A.V. & Caines, L.A. (1974) Nutrient chemistry of Loch Leven, Kinross. Proceedings of the Royal Society of Edinburgh (B). 74, 101-121.

Horne, A.J. & Fogg, G.E. (1970) Nitrogen fixation in some English Lakes. Proceedings of the Royal Society of London (B) 175 351-

Horne, A.J. & Viner, A.B. (1971) Nitrogen fixation and its significance in tropical Lake George, Uganda. Nature. 232, 417-418.

Howard, D.L., Frea, J.I., Pfister, R.M. & Dugan, P.R. (1970) Biological nitrogen fixation in Lake Erie. Science. 169, 61-62.

Hubley, J.H. (1975) Studies on the physiology of methane-utilizing bacteria. Ph D Thesis, University of Edinburgh.

Hubley, J.H., Thomson, A.W. & Wilkinson, J.F. (1975) Specific inhibitors of methane oxidation in Methylosinus trichosporium. Archives of Microbiology. 102, 199-202.

Huser, R. (1965) Zur mikrobiologischen luftstick-stoffbindung in Buchenstreu und Buchenmull. Plant and Soil. 23, 236-246.

Hutchinson, G.E. (1957) A treatise on limnology I. Geography, physics and chemistry. John Wiley & Sons, New York.

Jupp, B.P. & Spence, D.H.N. (1974) Macrophyte studies. In Loch Leven Research Group Annual Report (Institute of Terrestrial Ecology, 12 Hope Terrace, Edinburgh). 13-18.

Jupp, B.P., Spence, D.H.N. & Britton, R.H. (1974) The distribution and production of submerged macrophytes in Loch Leven, Kinross. Proceedings of the Royal Society of Edinburgh (B). 74, 195-208.

Katznelson, H. (1965) Nature and importance of the rhizosphere. In Baker, K.F. and Snyder, W.C. (eds). Ecology of soil-borne plant pathogens. A prelude to biological control. John Murray, London. 187-209.

Kavanagh, E.P. & Postgate, J.R. (1970) Absorphin and release of hydrocarbons by rubber closures: A source of error in some biological assays. Laboratory Practice. 19, 159-160.

Keeney, D.R. (1973) The nitrogen cycle in sediment-water systems. Journal of Environmental Quality. 2, 15-29.

Keeney, D.R., Chen, R.L. & Graetz, D.A. (1971) Denitrification and nitrate reduction in sediments! Importance to the nitrogen budget of lakes. Nature. 233, 66-67.

Keirn, M.A. & Brezonik, P.L. (1971) Nitrogen fixation by bacteria in Lake Mize Florida and in some lacustrine sediments. Limnology and Oceanography. 16, 720-731.

Knowles, R. (in press) The significance of asymbiotic dinitrogen fixation by bacteria. In R.W.F. Hardy et al (eds) Dinitrogen fixation Wiley - Interscience.

Macgregor, A.N. & Keeney, D.R. (1973) Acetylene-reduction assay of anaerobic nitrogen fixation by sediments of selected Wisconsin lakes. Journal of Environmental Quality. 2, 438-440.

Macgregor, A.N., Keeney, D.R. & Chen, K.L. (1973) Nitrogen fixation in lake sediments: contribution to nitrogen budget of Lake Mendota. Environmental Letters. 4, 21-26.

McRoy, C.P., Goering, J.J. & Chaney, B. (1973) Nitrogen fixation associated with sea grasses. Limnology and Oceanography. 18, 998-1002.

Magdoff, F.R. & Bouldin, D.R. (1970) Nitrogen fixation in submerged soil-sand-energy material media and the aerobic-anaerobic interface. Plant and Soil. 33, 49-61.

Marsho, T.V., Burchard, R.P. & Fleming, R. (1975) Nitrogen fixation in the Rhode River estuary of Chesapeake Bay. Canadian Journal of Microbiology. 21, 1348-1356.

Morgan, N.V. (1970) Changes in the fauna and flora of a nutrient enriched lake. Hydrobiologia. 35, 545-553.

Mortimer, C.H. (1938) The nitrogen balance of large bodies of water. Public Health Services Congress 1938, paper no. 16a. 3-11.

Mortimer, C.H. (1941) The exchange of dissolved substances between mud and water in lakes. Journal of Ecology. 29, 280-329.

Mortimer, C.H. (1942) The exchange of dissolved substances between mud and water in lakes. Journal of Ecology. 30, 147-201.

Mortimer, C.H. (1971) Chemical exchanges between sediments and water in the Great Lakes - speculations on probable regulatory mechanisms. Limnology and Oceanography. 16, 387-404.

Nicholas, D.J.D. (1965) Influence of the rhizosphere on the mineral nutrition of the plant. In Baker, K.F. and Snyder, W.C. (eds). Ecology of soil borne plant pathogens. A prelude to biological control. John Murray, London. 210-217.

Oremland, R.S. & Taylor, B.F. (1975) Inhibition of methanogenesis in marine sediments by acetylene and ethylene: validity of the acetylene reduction assay for anaerobic microcosms. Applied Microbiology. 30, 707-709.

Patriquin, D. & Knowles, R. (1972) Nitrogen fixation in the rhizosphere of marine angiosperms. Marine Biology. 16, 49-58.

Postgate, J.R. (1971a) Relevant aspects of the physiological chemistry of nitrogen fixation In Hughes, D.E. and Rose, A.H. Twenty-first symposium of the Society for General Microbiology. Microbes and Biological Productivity. 287-307.

Postgate, J.R. (1971b) Fixation by free-living microbes: physiology. In The Chemistry and Biochemistry of Nitrogen Fixation. Plenum Press.

Primrose, S.B. (1975) Factors affecting ethylene production by Escherichia coli. Paper given at 74th Ordinary Meeting of Society for General Microbiology.

Primrose, S.B. & Dilworth, M.J. (1975) Ethylene production by bacteria. Paper given at 74th Ordinary Meeting of Society for General Microbiology.

Reuszner, H.W. (1933) Marine bacteria and their role in the cycle of life in the sea III The distribution of bacteria in the ocean waters and muds about Cape Cod. Biological Bulletin. 65, 480-497.

Rice, W.A. & Paul, E.A. (1971) The acetylene reduction assay for measuring nitrogen fixation in waterlogged soil. Canadian Journal of Mircobiology. 17, 1049-1056.

Rinaudo, G., Balandreau, J. & Dommergues, Y. (1971) Algal and bacterial non-symbiotic nitrogen fixation in paddy soils. In Lie, T.A. and Mulder, E.G. (eds). Biological nitrogen fixation in natural and agricultural habitats.

Rovira, A.D. (1965) Plant root exudates and their influence upon soil mircoorganisms. In Baker, K.F. and Snyder, W.C. (eds). Ecology of Soil-Borne Plant Pathogens. Prelude to biological control. John Murray, London. 170-186.

Rusness, D. & Burris, R.M. (1970) Acetylene reduction nitrogen fixation in Wisconsin lakes. Limnology and Oceanography. 15, 808-813.

Schollhorn, R. & Burris, R.M. (1966) Study of intermediates in nitrogen fixation. Federation Proceedings. 25, 710.

Schollhorn, R. & Burris, R.M. (1967) Acetylene as a competitive inhibitor of N_2 fixation. Proceedings of the National Academy of Sciences of the U.S.A.

Seddon, B. (1972) Aquatic macrophytes as limnological indications. Freshwater Biology. 2, 107-130.

Smith, I.R. (1974) The structure and physical environment of Loch Leven, Scotland. Proceedings of the Royal Society of Edinburgh (B). 74, 81-100.

Spiff, E.D. & Odu, C.T.I. (1973) Acetylene reduction by Beijerinckia under various partial pressures of oxygen and acetylene. Journal of General Microbiology. 78, 207-209.

Stewart, W.D.P., Fitzgerald, G.P. & Burris, R.M. (1967) In situ studies on Nitrogen-fixation using the acetylene reduction technique. Proceedings of the National Academy of Sciences of the U.S.A. 58, 2071-2078.

Stewart, W.D.P., Haystead, A. & Pearson, H.W. (1969) Nitrogenase activity in heterocysts of blue-green algae. Nature. 224, 226-228.

Stewart, W.D.P. (1969) Biological and ecological aspects of nitrogen fixation by free-living microorganisms. Proceedings of the Royal Society of London (B). 172, 367-388.

Stewart, W.D.P., Mague, T., Fitzgerald, G.P. & Burris, R.M. (1971) Nitrogenase activity in Wisconsin lakes of differing degrees of eutrophication. New Phytology. 70, 497-509.

* see bottom of page

Terry, R.E. & Nelson, D.W. (1975) Factors influencing nitrate transformations in sediments. Journal of Environmental Quality. 4, 549-554.

Vollenweider, R.A. (1968) Scientific fundamentals of the eutrophication of lakes flowing waters with particular reference to nitrogen and phosphorus as factors in eutrophication. In DAS/CS1/68. 27. Paris: Organisation for Economic Cooperation and Development.

Watanabe, A. & Yamamoto, Y. (1967) Heterotrophic nitrogen fixation by blue-green algae Anabaenopsis circularis. Nature. 214, 738.

* Sylvester-Bradley, R, Döbereiner, J, Holding A J (1974) Acetylene reduction technique : the effect of acetylene on ethylene uptake by soils and lake sediments. Bull. Int. Inf. Biol. Sol. 19 5-9 (enclosed at back of thesis)

Sylvester-Bradley, R (preprint enclosed at back of thesis) Isolation of acetylene reducing spirilla from the roots of Potamogeton filiformis from Loch Leven (Kinross).

Werner, D., Evans, M.J. & Seidler, R.J. (1974) Facultatively anaerobic nitrogen-fixing bacteria from the marine environment. Canadian Journal of Microbiology. 20, 59-64.

Whitney, D.E., Woodwell, G.M. & Howarth, R.W. (1975) Nitrogen fixation in Flax Pond: A Long Island salt marsh. Limnology and Oceanography. 20, 640-643.

Whittenbury, R., Dalton, M., Eccleston, M. & Reed, H.L. (1975) The different types of methane oxidizing bacteria and some of their more unusual properties. In Microbial Growth on C_1-compounds Society of Fermentation Technology Japan. 1-9.

Whittenbury, R., Phillips, K.C. & Wilkinson, J.F. (1970) Enrichment, isolation and some properties of methane-utilizing bacteria. Journal of General Microbiology. 61, 205-218.

Yoshida, T. & Ancajas, R.R. (1971) Nitrogen fixation by bacteria in the root zone of rice. Proceedings of the Soil Science Society of America. 35, 156-157.

Yoshida, Y. & Broadbent, F.E. (1975) Movement of atmospheric nitrogen in rice plants. Soil Science. 120, 288-291.

Zuberer, D.A. & Silver, W.S. (in press) Mangrove associated nitrogen fixation. to be published in Proceedings of First International Symposium on biology and management of mangroves. U.S. Government Printing Office.

NOTES TECHNIQUES - TECHNICAL PAPERS - TECHNISCHE NOTIZEN

ACETYLENE REDUCTION TECHNIQUE : THE EFFECT OF ACETYLENE ON ETHYLENE UPTAKE BY SOILS AND LAKE SEDIMENTS

R.Mary SYLVESTER-BRADLEY*, Johanna DOBEREINER**, A.J. HOLDING

Nitrogenase, the enzyme complex which catalyses the fixation of atmospheric nitrogen, has several alternative substrates, one of which is acetylene. Estimation of the rate of reduction of acetylene (C_2H_2) to ethylene (C_2H_4) has been suggested as a method for the assay of nitrogenase activity in field samples (HARDY et al., 1968), and is now widely applied. However, it has been reported that rice paddy soils (YOSHIDA, 1971; YOSHIDA and ANCAJAS, 1971; YOSHIDA, pers.comm.) and non-water-logged soils (ABELES et al., 1971; SMITH et al.,in press) can absorb ethylene from the atmosphere. If this uptake also took place during nitrogenase assays of field samples by the acetylene reduction method, rates of ethylene production would be underestimated. It is necessary to obtain reliable estimates of ethylene production rates in the nitrogen fixation studies being undertaken in this laboratory, and so preliminary (unpublished) investigations were made to show wether a variety of lake sediments from the English Lake District, and Loch Leven, Kinross, Scotland, absorbed ethylene from the gas phase. It was found that considerable ethylene absorption did occur. Therefore, in the study described here, some soils and Loch Leven sediments were investigated further. Rates of ethylene uptake were observed in the presence and absence of acetylene under aerobic and anaerobic conditions.

MATERIALS AND METHODS

The soil and lake sediment samples are described in the figure legends. Ten grams of wet soil or sediment were added to McCartney bottles (28 ml) fitted with Suba-seal stoppers (obtainable from William

Present addresses :

* Department of Microbiology, School of Agriculture, University of Edinburgh, West Mains Road, EDINBURGH, EH9 3JG, Scotland

** Instituto de Pesquisa Agropecuaria Centro Sul, Km47 via Campo Grande ZC-26, Brazil

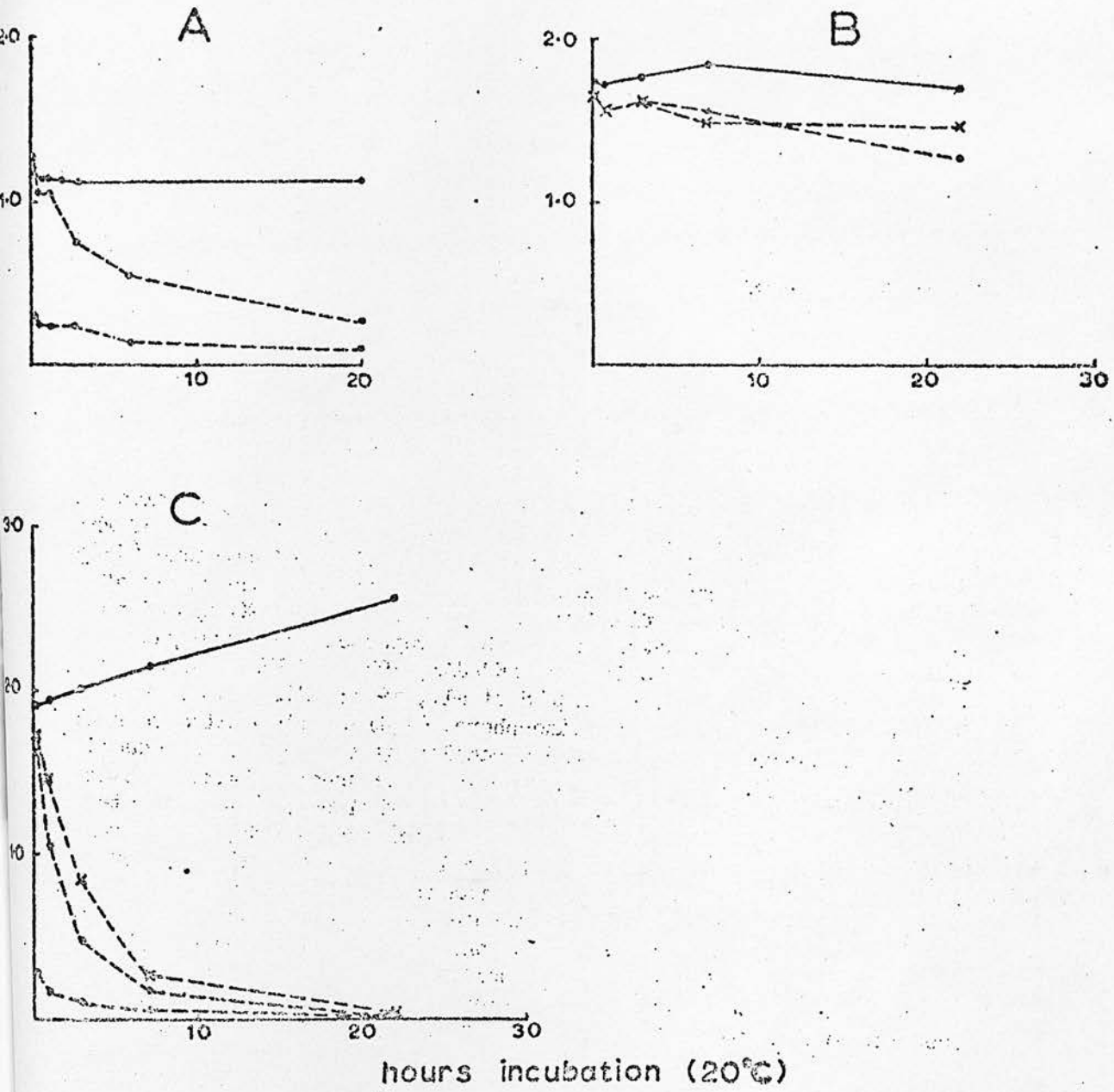


Fig. 1 : Total ethylene in the gas phase over three Loch Leven sediments (surface samples) in presence (——) and absence (- - -) of 10% v/v acetylene, under anaerobic (•) and initially aerobic (x) conditions. Results are means of three replicates corrected against control containing ethylene but no sediment .

Sediment (A) - 0.5 m below water surface : mixed clay and sand
Sediment (B) - 0.5 m below water surface : clay
Sediment (C) - 20 m below water surface.

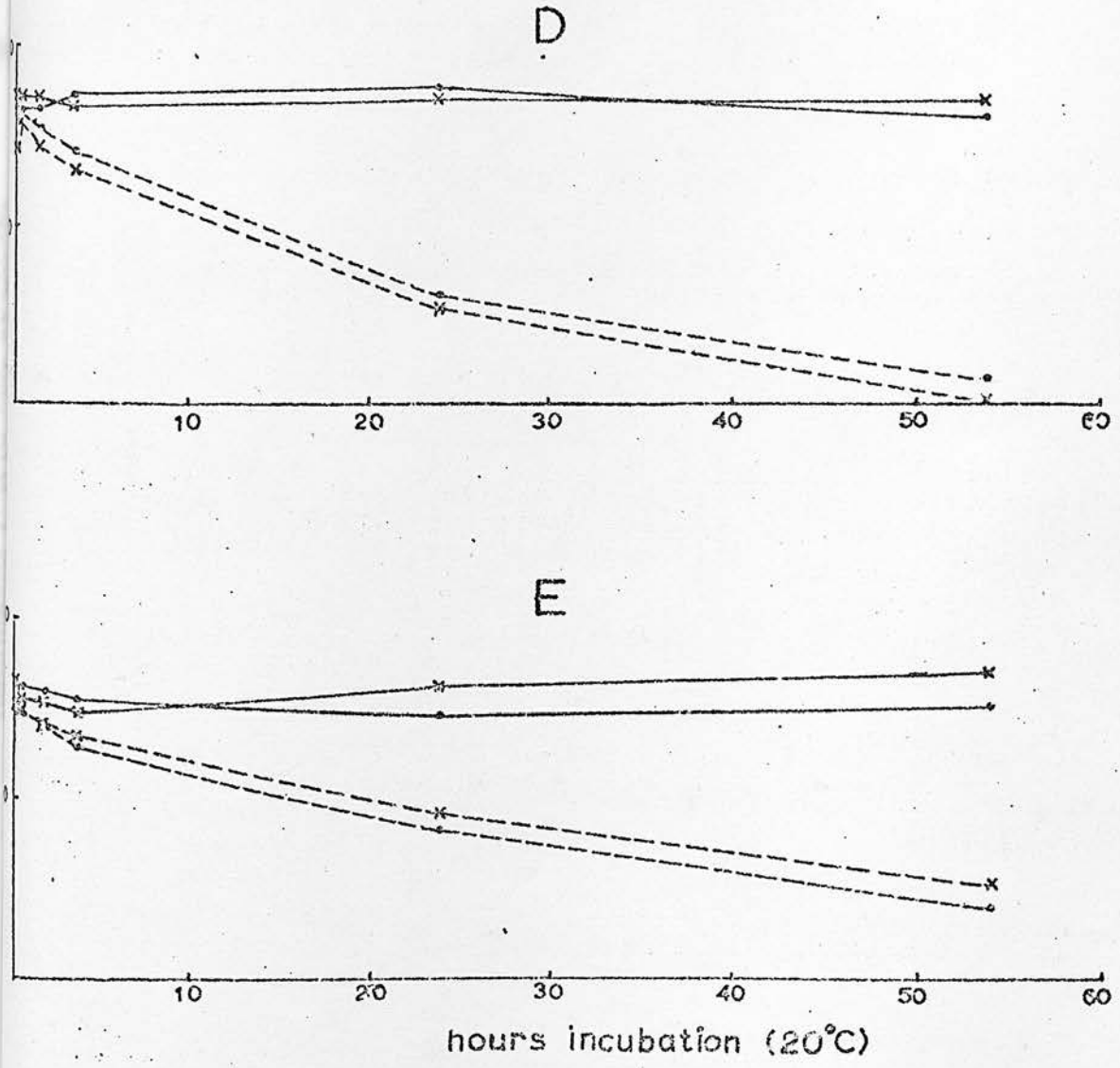


Fig. 2 : Total ethylene in gas phase over two soils (surface samples):

Soil (D) - Basin peat
Soil (E) - Darvel (freely drained Brown Earth).

Experimental conditions as for Fig. 1 .

Freeman & Sons Ltd, Suba-seal Works, Staincross, Barnsley, Yorkshire). In the anaerobic treatments the bottles were evacuated to between 20 and 100 mm Hg, and the gas phase replaced by oxygen-free nitrogen, three times. Aerobic treatments were not evacuated. Appropriate dilutions of ethylene and acetylene were made in flasks of known volume fitted with Suba-seal stoppers. Then, at zero time, ethylene was injected into the prepared McCartney bottles to give concentrations shown in the figures. In the acetylene treatments, pure or diluted acetylene was also added, to give concentrations of about 10% or 0.0025% v/v. The bottles were incubated at room temperature (20°C). The gas phase was sampled at intervals and the ethylene and acetylene concentrations measured in a Pye gas chromatograph with a flame ionization detector, and a 6 mm x 1.5 m glass column packed with Porapak R, at 70°C, and N_2 as carrier gas (flow rate 25 ml/min).

RESULTS AND DISCUSSION

1. In the absence of acetylene

Figs. 1 and 2 show that ethylene was lost from the gas phase of all the soil and sediment samples, to an extent which would seriously affect estimation of ethylene produced by acetylene reduction. The rate of decrease depended on the initial concentration of ethylene, and was particularly high with deep Loch Leven sediment, where after 2 hours more than half the ethylene had disappeared. Ethylene did not disappear from control treatments containing no soil or sediment so it cannot have been adsorbed onto the rubber bungs (KAVANACH and POSTGATE, 1970), or have leaked away. Soil and sediment samples which have been autoclaved (30 mins at 15 lb/in^2) also showed no loss of ethylene (results not shown). Such heat sensitivity might indicate that the ethylene uptake was due to biological decomposition in the samples. On the other hand, it seems to be independent of the oxygen tension in the gas phase (see figures). This would make a biological process unlikely.

2. In the presence of acetylene

ZOBELL's (1950) description of marine bacteria which "oxidised ethane, utilised ethylene more rapidly and attacked acetylene more rapidly than either", suggests that if the process of ethylene uptake were microbiological, it might be affected by the presence of acetylene. Obviously this would have direct implications for the acetylene reduction technique. Figs. 1 and 2 show that when 10% v/v acetylene was added as well as the ethylene, no ethylene uptake was observed. In fact, in the deep lake sediment there was an increase, which could be attributed to nitrogenase activity. In other treatments acetylene was added at the same concentration as the ethylene (about 0.0025% v/v), and even at such a low level no ethylene uptake was observed (results not shown).

Two possible alternative explanations for ethylene uptake not being observed when acetylene is present are:

a)- that ethylene uptake is inhibited by acetylene,

or

b)- that reduction of acetylene to ethylene compensates for the loss of ethylene observed in the absence of acetylene.

With an acetylene concentration as low as 0.0025% one would expect a much lower rate of acetylene reduction than with a 10% concentration, b) would therefore seem to be an unlikely explanation for the results obtained.

It appears, then, that acetylene must in some way inhibit ethylene uptake. If so, the inhibition is complete within the sensitivity range of the acetylene reduction assay, and occurs at concentrations of acetylene far below those normally used. Such a process might well be controlled by micro-organisms, as is suggested by ZoBell's observations (op. cit.).

Thus, the results suggest that although the samples tested can remove ethylene from the gas phase, they do not do so during the acetylene reduction assay.

References

ABELES, F.B.; L.E. CRAKER; L.E. FORRENCE, G.R. LEATHER, 1971, Fate of air pollutants: removal of ethylene, sulfur dioxide, and nitrogen dioxide by soil. Science, 173, 914-916

HARDY, R.W.F.; R.D. HOLSTEN; E.K. JACKSON; R.C. BURNS, 1968, The acetylene-ethylene assay for nitrogen fixation: laboratory and field evaluation. Pl. Physiol., 43, 1185-1207

KAVANAGH E.P.; J.R. POSTGATE, 1970, Absorption and release of hydrocarbons by rubber closures: a source of error in some biological assays. Lab. Prac., 19, 159-160

SMITH, K.A.; J.M. BREMNER; TABATABAI, M.A., 1973. Sorption of gaseous atmospheric pollutants by soils. Soil Science (in press).

YOSHIDA, T.; R.R. ANCAJAS, 1971. Nitrogen-fixation by bacteria in the root zone of rice. Soil Sci. Soc. Amer. Proc., 35, 156-157

YOSHIDA, T., 1971, Research Results Report, Soil Microbiology. International Rice Research Institute, Los Banos, Philippines

ZOBELL, C.E., 1950. Assimilation of hydrocarbons by microorganisms. Adv. Enzymol., 10, 443-486

ISOLATION OF ACETYLENE REDUCING SPIRILLA FROM THE ROOTS OF POTAMOGETON FILIFORMIS FROM LOCH LEVEN (KINROSS)

Potamogeton filiformis is a submerged macrophyte of particular ecological importance in Loch Leven, (Kinross). Its percentage cover has increased considerably over recent years despite an overall decline of aquatic macrophytes in the loch (Jupp, Spence & Britton, 1974; Britton, 1974). Part of this decline has been attributed to an increase in the productivity of certain types of phytoplankton (e.g. heterocystous blue-green algae) following eutrophication. The phytoplankton blooms decrease the light available to submerged macrophytes (Jupp et al, 1974). The wildfowl, for which Loch Leven is famous, have also shown recent population changes. This may be a result of the decrease in macrophytes (Allison & Newton, 1974).

It has been reported that significant levels of nitrogen fixation (measured by $^{15}N_2$) and/or acetylene reduction (Hardy, Holsten, Jackson & Burns, 1968) occur on the roots of aquatic macrophytes (Patriquin & Knowles, 1972; Bristow, 1974; Zuberer & Silver, in press), and on the roots of rice seedlings growing in waterlogged soil (Dommergues, Balandreau, Rinaudo & Weinhard, 1973), and this activity is attributed to asymbiotic bacteria. However, of the various species of nitrogen fixer isolated from these systems, none have been shown to be responsible for the nitrogenase activity. Enrichment cultures of nitrogen fixers are selective for particular species; the species responsible would remain undetected unless the appropriate conditions were chosen. In the study reported here nitrogen fixing bacteria from the roots of P. filiformis were investigated, because it seemed that the recent spreading of the plant might be an effect of nitrogen fixation. Also, to estimate the quantity of nitrogen fixation occurring in any system, it is necessary to know the characteristics of the organisms responsible.

Similar methods to those of Day & Dobereiner (1976) were used. Small (0.5 to 1 cm long) pieces of freshly sampled P. filiformis roots were washed and placed in 1 cm deep nitrogen-free semi-solid malate medium in 1 oz vials (for media see legend to Table 1). Invariably, after 1 day's incubation under air at 30°C, sub-surface pellicles of microbial growth appeared. These enrichment cultures reduced acetylene even after the root had been removed (acetylene reduction was carried out as described in legend to Table 1). Phase microscopic examination

of the cultures revealed very motile spiral-shaped organisms 2-3 x 5-10 u in size, containing many refractile bodies.

Purification of the organisms responsible for the acetylene reduction in these cultures proved very difficult until a method of inoculating enrichment cultures into water or buffered saline, mixing with a Whirlimix, and pour- rather than streak-plating was adopted; 0.05% yeast extract or vitamin-free casamino acids was required in the otherwise nitrogen-free malate medium.

By this method five pure cultures of bacteria which consistently reduce acetylene were obtained. The five cultures originate from samples of P. filiformis collected from different sites in the loch. The bacteria are much smaller than the organisms observed in the original enrichment cultures (0.5 - 1.0 x 3 u), even more motile, and do not contain refractile bodies. They have half to one spiral turn per cell and sometimes join together to form spiral chains. They form white opaque colonies in pour-plates of malate medium containing 0.05% vitamin-free casamino acids (VfC) and 1.5% Noble agar, and buff-coloured translucent colonies on nutrient agar. They are Gram-negative. Of the Spirillum species described in Bergey's Manual of Determinative Bacteriology (1957), they are most closely related to S. lipoferum, and many of their characteristics correlate with those observed by Beijerinck (1925).

Some characteristics of the spirilla in pure culture were investigated further. Under air and at 30°C they do not grow in completely nitrogen-free malate medium (Medium 1), but grow in a sub-surface pellicle and reduce acetylene vigorously in semi-solid malate medium containing 0.05% VfC (Medium 2; see Table 1). They do not grow in liquid Medium 2. In semi-solid malate medium containing no VfC but 0.1% $(NH_4)_2NO_3$ (Medium 3), they grow nearer the surface and do not reduce acetylene. They grow well in liquid Medium 3 (although in wet mounts a micro-aerotactic response as described by Wells & Krieg (1965) for S. volutans is observed). This apparent decrease in oxygen sensitivity and concomitant lack of acetylene reduction in Medium 3 is as expected for aerobic nitrogen-fixing bacteria: in other such species nitrogenase synthesis is inhibited by fixed nitrogen, and continuous cultures are very oxygen sensitive when fixing nitrogen (Hill, Drozd & Postgate, 1972).

VfC are only needed for growth in otherwise nitrogen-free media. The need is not satisfied by 0.01% $(NH_4)_2NO_3$ or a wide range of single

amino acids. However, when the air over semi-solid Medium 1 was replaced by 2% O_2 in N_2, the spirilla grew at the surface and reduced acetylene almost as well as in Medium 2 (see Table 1). Thus, apparently, the role of VfC is to enable nitrogen-fixing spirilla to overcome oxygen toxicity. N_2 possibly becomes limiting just below the surface of the agar; under air the bacteria would be forced to grow at super-optimal pO_2 in order to obtain sufficient N_2. A similar protective mechanism of yeast extract has been observed in Mycobacterium flavum 301 (Biggins & Postgate, 1969), and of vitamin-free casamino acids in Klebsiella pneumoniae (Hill, 1975). Von Bulow & Dobereiner (1975) and Dobereiner & Day (in press) have isolated S. lipoferum from the roots of maize and Digitaria decumbens in Brazil, and their results imply that it supplies a large proportion of the plants' nitrogen. Their isolates grow under air on completely nitrogen-free media, they will grow on glucose, although not in nitrogen-free media (Day & Dobereiner, 1976), and a pink pigment is formed in older cultures. The isolates described here do not grow on glucose with $(NH_4)_2NO_3$ under air (Medium 4), and do not grow better on glucose plus VfC (Medium 5) under air than can be explained by their growth on VfC alone (Medium 6) (see Table 1). No pigment has been observed. Thus, although they are similar in some ways to the maize and Digitaria isolates, they seem to differ in their requirement for VfC and inability to grow on glucose under air, and in pigment formation. The nitrogen fixing spirilla described by Becking (1963) did not grow in the absence of yeast extract, and may have been the same as the isolates described here. Thus these preliminary data support the suggestion of Dobereiner & Day (in press) that there is more than one form of S. lipoferum.

Some unpublished experiments (Sylvester-Bradley, in preparation) have shown that washed P. filiformis roots from Loch Leven can reduce acetylene at an optimum oxygen concentration (v/v) above 0% and below 5% (average rates observed at lake temperature up to 90 nmoles C_2H_2/g dry roots/h) in the dark. The roots of P. filiformis are encrusted with red ferric iron compounds which contrast with the black or grey of the clay they are embedded in. The plant also possesses an aerenchyma. This is a channel of tissue commonly found in aquatic plants through which air passes from the shoots to the roots. Aeration of the root zone has been observed and measured in many aquatic and marsh plants (Armstrong, 1972). It can therefore reasonably be assumed that the root zone of

P. filiformis is aerobic. These observations, together with the fact that all root pieces enriched produce sub-surface pellicles of microbial growth which reduce acetylene, indicate that the spirillum isolated may well be responsible for nitrogen fixation in the roots of P. filiformis Further studies using $^{15}N_2$ are needed to estimate the proportion of nitrogen supplied to the plant by nitrogen fixation. The spirilla isolated by Dobereiner & Day (in press) appeared to be growing within inner root cortex cells. The spirilla described here were isolated from washed roots, and thus it seems that they might also be found to be intracellular.

Some of the acetylene reducing activity described by Patriquin & Knowles (1972) was higher under aerobic than anaerobic conditions, and it is possible that this was due to spirilla. Thus investigations into the distribution of nitrogen fixing spirilla on plant roots in aquatic ecosystems might reveal that they are of considerable significance.

Rosemary Sylvester-Bradley,
Department of Microbiology,
School of Agriculture,
University of Edinburgh,
West Mains Road, Edinburgh, EH9 3JG.

I gratefully acknowledge the collaboration of Dr. J. Dobereiner at the initiation of this project, and the support of a N.E.R.C. research studentship at the University of Edinburgh, supervised by Dr. A.J. Holding.

References.

Allison, A. & Newton, I. (1974) Waterfowl at Loch Leven, Kinross. Proc. R. Soc. Edinburgh (B), 74, 365-381.

Armstrong, W. (1972) A re-examination of the functional significance of aerenchyma Physiologia Pl., 27, 173-177.

Becking, J.H. (1963) Fixation of molecular nitrogen by an aerobic Vibrio or Spirillum. Antonie van Leuvenhoek 29, 326.

Beijerinck, M.W. (1925) Uber ein Spirillum, welches freien Stickstoff binden kann? Zentbl. Bakt. Parasitkde. Abt II, 63, 353-359.

Biggins, D.R. & Postgate, J.R. (1969) Nitrogen fixation by cultures and cell-free extracts of Mycobacterium flavum 301. J. gen. Microbiol., 56, 181-193.

Breed, R.S., Murray, E.G.D., Smith, N.R. (1957) Bergey's Manual of Determinative Bacteriology, (7th Edition), Williams & Wilkins Co., Baltimore,.

Bristow, J.M. (1974) Nitrogen fixation in the rhizosphere of freshwater angiosperms. Can. J. Bot., 52, 217-221.

Britton, R.H. (1974) Factors affecting the distribution and productivity of emergent vegetation at Loch Leven, Kinross. Proc. R. Soc., Edinburgh (B), 74, 209-218.

Day, J.M., Dobereiner, J. (1976) Physiological aspects of N_2-fixation by a Spirillum from Digitaria roots. Soil Biol. Biochem. 8, 45-50.

Dobereiner, J. & Day, J.M. (in press) Associated symbioses in tropical grasses: characterzation of microorganisms and dinitrogen fixing sites. In International Symposium on N_2 fixation - inter-disciplinary discussions, June 3-7, 1974. (Washington State University, Pullman, Washington).

Dommergues, Y., Balandreau, J., Rinaudo, G., Weinhard, P. (1973) Non-symbiotic nitrogen fixation in the rhizospheres of rice, maize, and different tropical grasses. Soil Biol. & Biochem., 5, 83-89.

Hardy, R.W.F., Holsten, R.D., Jackson, E.K. & Burns, R.C. (1968) The acetylene-ethylene assay for N_2 fixation: laboratory and field evaluation. Pl. Physiol., 43, 1185-1207.

Hill, S. (1975) Acetylene reduction by Klebsiella pneumoniae in air related to colony dimorphism on low fixed nitrogen. J. gen. Microbiol., 91, 207-209.

Hill, S., Drozd, J.W. & Postgate, J.R. (1972) Environmental effects on the growth of nitrogen-fixing bacteria. J. appl. Chem. Biotechnol., 22, 541-558.

Jupp, B.P., Spence, D.H.N. & Britton, R.H. (1974) The distribution and production of submerged macrophytes in Loch Leven, Kinross. Proc. R. Soc., Edinburgh. (B), 74, 195-208.

Patriquin, D. & Knowles, R. (1972) Nitrogen fixation in the rhizosphere of marine angiosperms. Marine Biol., 16, 49-58.

Sylvester-Bradley, R. (in preparation) Investigations into the measurement of nitrogen fixation in freshwater lake sediments. Ph.D. Thesis, University of Edinburgh.

Von Bulow, J.F.W. & Dobereiner, J. (1975) Potential for nitrogen fixation in maize genotypes in Brazil. Proc. natn. Acad. Sci. U.S.A. 72, 2389-2393.

Wells, J.S. & Krieg, N.R. (1965) Cultivation of spirillum volutans in a bacteria-free environment. J. Bacteriol., 90, 817-818.

Zuberer, D.A., Silver, W.S. (in press) Mangrove associated nitrogen fixation. In Proceedings of the first international symposium on biology and management of mangroves. U.S. Government Printing Office.

Table 1

	Additions to basic semi-solid Medium:		Visible Growth	Acetylene reduction
der ir	Malate only	(1)	-	0
	Malate + VfC	(2)	+++	80
	Malate + 0.1% $(NH_4)_2NO_3$	(3)	+++	0
	Glucose + 0.1% $(NH_4)_2NO_3$	(4)	-	0
	Glucose + VfC	(5)	+	0
	VfC only	(6)	+	0
der O_2 N_2	Malate only	(1)	+++	60
	Malate + VfC	(2)	+++	80

Legend to Table 1

Relative growth and acetylene reduction of five Spirillum isolates in various semi-solid media. Washed suspensions of cells of five isolates grown in liquid malate plus $(NH_4)_2NO_3$ medium were inoculated into 2 ml semi-solid medium (see below) in 7.5 ml vials, and incubated for exactly one day at 30°C. The rate of acetylene reduction was then determined as follows: the caps were screwed down tightly and 10% C_2H_2 was added; after 1-2 hours incubation at 30°C the gas phase was analysed for ethylene by gas chromatography on a Pye 104 gas chromatograph with flame ionization detector; a 5 ft column packed with Porapak R, oxygen-free nitrogen carrier gas flow rate 34 mls/min, H_2 15 lb/in^2, air 20 lb/in^2, oven temperature 50°C. Controls without acetylene contained no ethylene. Ethylene contaminant in the acetylene was subtracted. The gas chromatograph was calibrated using dilutions of pure C_2H_4. It is not possible to enumerate cells in semi-solid media; results are therefore expressed as nmoles C_2H_4 produced per culture per hour (average of five cultures).

Basic medium (adapted from Dobereiner & Day, 1976):

0.4 g KH_2PO_4; 0.1 g K_2HPO_4; 0.2 g $MgSO_4$ $7H_2O$; 0.1 g NaCl;

0.02 g $CaCl_2$; 0.01 g $FeCl_3$; 0.002 g $NaMoO_4.2\ H_2O$; 1000 ml dist water.

Additions (one or combinations of): 0.5% Na malate; 0.5% glucose; 0.05% Difco Bacto vitamin-free casamino acids (VfC); 0.1% or 0.01% $(NH_4)_2NO_3$; 3.5 g (semi-solid) or 15 g (for pour-plates) Difco Bacto Special Noble agar; 0.05% yeast extract (in pour-plates).